
ESSENTIAL IVF

Basic Research and Clinical Applications

edited by
JONATHAN VAN BLERKOM
LINDA GREGORY



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TABLE OF CONTENTS

List of Contributors.....	ix
Preface.....	xv
CHAPTER ONE	
THE SYNTHETIC AND SECRETORY BEHAVIORS (NONSTEROIDAL) OF OVARIAN FOLLICULAR GRANULOSA CELLS: PARALLELS TO CELLS OF THE ENDOTHELIAL CELL LINEAGE <i>Michael Antczak</i>1	
CHAPTER TWO	
OOCYTE-GRANULOSA CELL INTERACTIONS <i>David Albertini</i>43	
CHAPTER THREE	
PERI-FOLLICULAR VASCULARITY: A MARKER OF FOLLICULAR HETEROGENEITY AND OOCYTE COMPETENCE AND A PREDICTOR OF IMPLANTATION IN ASSISTED CONCEPTION CYCLES <i>Linda Gregory</i>59	
CHAPTER FOUR	
DO BIOCHEMICAL PREDICTORS OF IVF OUTCOME EXIST? <i>Anthony Michael</i>81	
CHAPTER FIVE	
GENETICS OF MALE INFERTILITY: EVOLUTION OF THE X AND Y CHROMOSOME AND TRANSMISSION OF MALE INFERTILITY TO FUTURE GENERATIONS <i>Sherman Silber</i>111	
CHAPTER SIX	
SPERM ANALYSIS AND PREPARATION UPDATE <i>Martine Nijls and Willem Ombelet</i>151	

CHAPTER SEVEN

PREPARATION OF SPERM FRACTIONS AND INDIVIDUAL SPERM WITH LOW LEVELS OF CHROMOSOMAL ANEUPLOIDIES FOR IVF AND ICSI

*Tamas Kovacs, Attila Jakab, Ertug Kovanci,
Zoltan Zavaczki, Denny Sakkas and Gabor Huszar.....179*

CHAPTER EIGHT

MATERNAL AGE AND OOCYTE COMPETENCE

Ursula Eichenlaub-Ritter and Fengyun Sun.....201

CHAPTER NINE

GENETIC DIAGNOSIS OF METAPHASE II OOCYTES

Yury Verlinsky and Anver Kuliev.....231

CHAPTER TEN

OOCYTE COMPETENCE AND IN VITRO MATURATION

Jennifer Cavilla and Geraldine Hartshorne.....241

CHAPTER ELEVEN

WHAT IS THE ROLE OF MITOCHONDRIA IN EMBRYO COMPETENCY?

Carol Brenner.....273

CHAPTER TWELVE

FUNDAMENTALS OF THE DESIGN OF CULTURE MEDIA THAT SUPPORT HUMAN PREIMPLANTATION DEVELOPMENT

John Biggers.....291

CHAPTER THIRTEEN

THE BIOLOGICAL BASIS OF OOCYTE AND EMBRYO COMPETENCE: MORPHODYNAMIC CRITERIA FOR EMBRYO SELECTION IN IN -VITRO FERTILIZATION

Lynette Scott.....333

CHAPTER FOURTEEN

THE ENIGMA OF FRAGMENTATION IN EARLY HUMAN EMBRYOS: POSSIBLE CAUSES AND CLINICAL RELEVANCE

Jonathan Van Blerkom.....377

CHAPTER FIFTEEN

BLASTOCYST TRANSFER UPDATE: PROS AND CONS

*Anna Veiga, José Torelló, Irene Boiso, Pedro Barri,
and Yves Ménézo*.....423

CHAPTER SIXTEEN

ASSISTED HATCHING IN CLINICAL IVF

Graham Wright and Amy Jones.....441

CHAPTER SEVENTEEN

PROSPECTS FOR OBTAINING VIABLE OOCYTES FROM CRYOPRESERVED OVARIAN TISSUE

Helen Picton.....465

CHAPTER EIGHTEEN

CRYOPRESERVATION IN HUMAN ASSISTED REPRODUCTION

Yves Ménézo and Pierre Guerin.....485

CHAPTER NINETEEN

REDUCING THE NUMBER OF EMBRYOS TO TRANSFER AFTER IVF/ICSI

Jan Gerris.....505

CHAPTER TWENTY

CAN AND SHOULD HUMAN EMBRYOS BE “RESCUED” FROM DEVELOPMENTAL DEMISE? METHODS AND BIOLOGICAL BASIS

Jim Cummins.....555

CHAPTER TWENTY-ONE

DETERMINATION OF ENDOMETRIAL STATUS AND THE IMPLANTATION WINDOW

Markku Seppälä and Bruce Lessey.....577

INDEX.....611

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PREFACE

There is no clearer testament to the importance and efficacy of in vitro fertilization in the treatment of the infertile patient than the fact that over one million babies have been born since its clinical introduction in 1978. The success of this worldwide endeavor has evolved to treat some of the formerly most intractable forms of infertility and requires individuals with different skills and insights whose activities are often compartmentalized into clinical, laboratory and research functions. The intent of Essential IVF is to present current issues in clinical IVF that encompass the varied activities of those engaged in this enterprise. By integrating clinical, basic research and laboratory-related aspects of human reproduction, readers with diverse interests should obtain a more complete understanding of the impact, importance and inter-relatedness of each in the progress of infertility treatment, and an appreciation of whether emerging technologies will or should contribute to this progress in the near future. The topics selected for this volume include research that has begun to explain the origins of differential follicular, gamete, embryo and uterine competence, and specific laboratory procedures and protocols that may have important clinical implications for the generation of developmentally viable embryos.

Human embryo research over the past 25 years has not only confirmed that the developmental potential of each embryo is unique, but more importantly, demonstrated how genetic and nongenetic factors for sperm and oocyte determine embryo competence well before fertilization. Several chapters deal with the origins of normal and compromised gametes and how those with high competence can be identified and isolated for fertilization. While the generation of high competence embryos is an essential aspect of infertility treatment with IVF, so also is the current emphasis on the avoidance of higher order gestations by limiting the number of embryos transferred. Here, chapters that discuss criteria used for progressive noninvasive evaluations of embryo development provide a current indication of the utility of morphology in competence assessment, and present outcome based results that indicate patient- and cycle-specific characteristics in which the transfer of one or two embryos should be considered. Several chapters describe research and clinical efforts on follicle and preantral oocyte culture and cryopreservation to preserve the fertility of certain patients, while others discuss whether invasive manipulations such as ooplasmic transfer and assisted hatching have merit in the treatment of the infertile patient.

The imperative to constantly improve outcome is the engine that drives this field of reproductive medicine, an engine fueled by the frequent introduction of major changes in clinical and laboratory protocols, often before their presumed benefits have been fully validated or a solid biological foundation established. In the same respect, whether current practices remain valid years after their introduction is not often addressed and in many IVF

programs, certain practices persist long after their actual efficacy has been questioned. For chapters that describe current practices in the laboratory management of embryos, the authors critically review the rationale, design and validity of studies that have been reported to improve outcome. These reviews should be of particular relevance to clinicians and laboratory personnel as they question whether existing protocols and suggested changes are currently warranted and if so, whether they should be applied universally or on a selected basis.

Through the efforts of the contributors, this volume provides both historical and current perspectives on practices common in human IVF. While no specific consensus leading to a 'standardization' of clinical and laboratory protocols was intended or is evident, owing to the unique and different experiences of each author, the chapters do provide guidance with which existing and newer protocols of gamete and embryo selection, culture, and competence assessment can be evaluated. The basic research on follicular, embryonic and uterine biology provides a glimpse of ongoing efforts directed at the identification of cellular, biochemical and physiological determinants suggested to be associated with normal gamete and embryo developmental potential and to be predictive of outcome. The possibility of rescuing developmentally compromised oocytes and embryos, as well as the cryopreservation and culture of small follicles and their corresponding oocytes is at an experimental stage. However, it is clear from the descriptions of current research efforts that technical and biological foundations for future clinical application are being established.

While it is apparent that basic and clinical studies described in this book come from very different directions and perspectives, they have two common goals, improving outcome for the infertile patient and the generation of healthy children. In this context, all involved in clinical IVF are cognizant of the fact that the patients being treated are both the infertile couple and the intended offspring, and it is to the achievement of these dual goals that the information presented in this book is intended.

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CHAPTER ONE

THE SYNTHETIC AND SECRETORY BEHAVIORS (NONSTEROIDAL) OF OVARIAN FOLLICULAR GRANULOSA CELLS: PARALLELS TO CELLS OF THE ENDOTHELIAL CELL LINEAGE

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INTRODUCTION

The cells of the ovarian follicular granulosa are most frequently referred to as epithelial cells. In general, descriptions of these cells focus primarily on two principle functions. One of these functions is to nurture and help maintain the integrity of the oocyte, from the inception of the follicle up to the point of ovulation, and perhaps even slightly beyond. A second function attributed to these cells is the production of steroid hormones, principally progesterone, essential for maintaining the pregnancy, in the event that an ovulated egg(s) becomes fertilized. This second objective is accomplished following the successful transformation of the ovulated follicle into a functional endocrine gland, the corpus luteum, and integration of the structure into the systemic circulation. There is little doubt, at this point, that the cells of the ovarian follicular granulosa perform each of these functions but there is growing evidence that the breadth of the capabilities, activities and functions of these cells may be much broader than originally anticipated. As the number of factors known to be produced by ovarian granulosa cells continues to increase, it becomes progressively more difficult to imagine exactly how each newly identified activity fits in with the biology of these cells, at least as far as it is currently understood and portrayed. It would be a simpler matter, in some respects, if the cells of the follicular granulosa were largely nonresponsive secretory cells. If the granulosa cells were incapable of responding to the majority of the factors they produce, it would be easy to postulate that one of the primary purposes for the factors synthesized and secreted by these cells was to effect the fate of events occurring outside the follicle. Specifically, events occurring within the surrounding thecal tissues, with particular emphasis on the behavior of the endothelial cells of the perifollicular capillary bed. However, in the majority of cases, there is

evidence that the cells of the follicular granulosa are responsive to the factors they produce (growth factors, cytokines, bioactive molecules), in as much as they have been shown to express the appropriate cellular receptors for the particular factors in question. The more carefully this issue is examined the greater the accumulated evidence that there are higher levels of complexity and function associated with the cells of the follicular granulosa than those previously reported and/or proposed. The exact nature and purpose for many of the known behaviors and activities of the follicular granulosa are still beyond our grasp. As a result, there is a compelling need to ask "why" these cells do the things they do if their primary objectives are simply nurturing and maintaining the integrity of the oocyte and producing progesterone. The need to ask "why" is compelling because the extent to which the behaviors of the follicular granulosa seem superfluous and unnecessary may very likely be an accurate measure of our current lack of understanding of the true character and functions of these cells. Perhaps most importantly, the need to ask "why" is compelling because the effect(s) the cells of the follicular granulosa exert on the oocyte may be far more fundamental and pervasive than accounted for by current models and dogmas regarding the follicle cells, their duties and their relevance. Yet, to answer the simple question "why" may not be so simple and it will almost certainly require some thinking outside the confines represented by the currently held paradigms regarding the nature of these cells and their behaviors.

The objective of this chapter is to provide an alternate perspective, to go beyond the limits delineated by today's widely held beliefs, impressions and perceptions regarding the nature and function(s) of mammalian follicular granulosa cells. What conclusions would be drawn concerning the nature, identity and activities of ovarian follicular granulosa cells were the commonly held dogmas set aside, at least for the time being, and more recent findings considered in their absence? What does new information acquired over the last decade or so suggest about the purpose, function and identity (cell lineage) of these cells and how might that fit in with, modify, or supersede known follicle cell biology, if true? If one of the primary purposes of all life is to beget new life, what type of cell(s) might be strategically placed within the follicle, with the oocyte, to optimize the functionality and fidelity of the system? What is the current state of the art with respect to mammalian evolution and the ovarian follicle – a quintessential player in the survival of each of the affected species? Do ovarian granulosa cells, though somewhat specialized, represent just another population of 'ordinary', somatic cells? Have the forces of evolution and the need for efficient, effective procreation selected an unusual and particularly capable somatic cell population(s) for duty in the follicle, to ensure the function and integrity of one of life's most fundamental goals – rebirth and regeneration? The purpose of this chapter is not to provide all the answers, nor to provide any answers, necessarily. The purpose of this chapter is to ask "why". The purpose of this chapter is to

present a novel perspective and corroborating evidence that appears to support the view proffered. The purpose of this chapter is to speculate and suggest, to think outside the box and to provide a novel perspective from which to ponder the cells of the ovarian follicular granulosa.

THE EPITHELIAL NATURE OF OVARIAN FOLLICULAR GRANULOSA CELLS

What does it mean to be described as an epithelial or epithelial-like cell, the designation most frequently assigned to the cells of the follicular granulosa? What are the parameters used to designate or identify a cell as epithelial? Is the assignment made on the basis of distinct morphological characteristics? Does the assignment imply derivation from the ectodermal/entodermal germ cell layer? Unfortunately, the answers to some of these questions are time and source dependent.

The word epithelium is derived from the roots “epi” meaning upon and “thele” meaning nipple. The term was first used to describe the translucent covering of the lips over the little “nipples” of connective tissue immediately beneath the surface, red in color because of their rich capillary investment (Ham, 1969). Initially, epithelium was used to designate cellular membranes covering or lining surfaces on or in the body, but in time the term also came to be used to designate the cells of glands which were derived from covering and lining membranes. The 1901 edition of Gray’s Anatomy suggested that epithelial cells, “as a rule”, form from the epiblast or hypoblast layers of early embryonic development (Gray, 1901). Today we know that while most forms of epithelium are derived from ectodermal or entodermal germ cell layers, some epithelial cells are also derived from the mesodermal germ cell layer (epithelium of the adrenal glands, urinary and genital systems), (Ham, 1969). As a result, today, the designation “epithelial” cannot be used, *a priori*, to suggest or determine germ cell derivation. Rather, the “epithelial” assignment is made on the basis of a series of morphological criteria – specifically, the associated microscopic structure, position and, to some extent, the function of the cells in question (Ham, 1969). Based on these characteristics, epithelial cells are grouped into several main classes, including: a) simple squamous, b) simple cuboidal, c) simple columnar, d) pseudostratified ciliated columnar, e) transitional, and f) stratified squamous.

While histology textbooks over the years have been in rough agreement concerning the description and morphological characteristics of epithelial

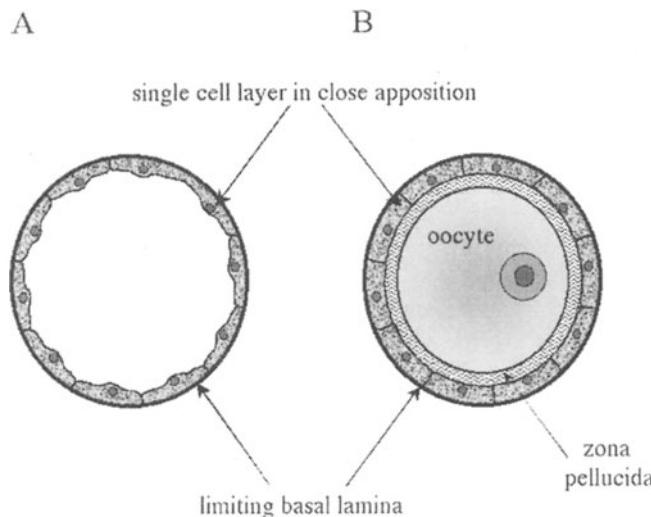


FIGURE 1.1. Schematic representation of a cross section of the simplest blood vessel, a capillary (A) and the most basic unit of mammalian reproductive function, the primordial follicle (B). Both structures are bounded by a limiting basal lamina, and contain a single layer of flattened, tightly apposed squamous cells. Noteably, the primordial follicle contains a single immature oocyte.

cells, considerable differences exists when addressing cells of the endothelium and mesothelium. Cells of the endothelium line the vessels of the vascular and lymphatic systems. Cells of the mesothelium line the body cavity and the organs that extend into them. Based on the criteria used to designate cells as epithelial, cells of the endothelium and mesothelium would both be classified as epithelial cells, in particular, simple squamous epithelium. However, for a considerable period of time, up until and including the present, there has been reluctance on the part of some to include endothelium and mesothelium within the family of epithelium, in large measure, it would appear, because of their mesodermal origin. For example, the 1901 version of Gray's Anatomy suggested that epithelium and endothelium should represent two distinct classes of cells (Gray, 1901). Today, some contemporary textbooks include cells of the endothelium and mesothelium as specific examples of epithelium (Hay, 1966; Copenhaver et al., 1971) while others exclude them from the family of epithelium, for a variety of tenuous reasons (Ham, 1969). The latter approach, excluding endothelial cells from the family of epithelial cells, is most unfortunate for a variety of reasons. For example, simple squamous epithelium is frequently described as an interdigitated arrangement of thin flat cells sitting atop a basement membrane that in concert form a continuous membranous sheet. Further, simple squamous epithelium is characterized as a cellular

arrangement designed to perform a filtering or dialyzing function. These descriptions are in good agreement with the functional and morphological features and properties of the endothelial cells that line the blood vessels of the body. A schematic representation of a cross section of the simplest of blood vessels, a capillary, is shown in figure 1.A. Of particular note, these same features and characteristics would be equally appropriate for a description of the functional and morphological characteristics of the granulosa cells of a primordial follicle, the basic unit of mammalian reproductive function, as shown in figure 1.1B. The obvious difference, and a substantial one at that, is the presence of the oocyte in the primordial follicle.

Finally, evidence has been recently presented suggesting that the cells of the follicular granulosa may represent a specialized endothelial-like cell population (Antczak and Van Blerkom, 2000). The similarities between follicle cells and endothelial cells and some possible rationales for why the choice of an endothelial or endothelial-like cell for the ovarian follicle would seem to make good biological sense are presented below.

THE SYNTHETIC AND SECRETORY BEHAVIORS OF OVARIAN FOLLICULAR GRANULOSA CELLS

If the cells of the ovarian follicular granulosa are simply just another somatic cell population(s) that associates with the oocyte prior to follicle formation and beyond, then how do these cells acquire their unusual complexity and their diverse capabilities *in situ*? Is this cell population transformed into something completely different from its predecessors/extrafollicular counterparts under the influences of the oocyte? Or, alternatively, does this cell population(s) enter the scene and associate with the egg very much prepared to perform the functions it was destined and uniquely selected to perform? Unfortunately, the answers to these questions remain outside the grasp of current understanding. However, well within current capabilities is a consideration of some of the synthetic and secretory activities currently attributed to ovarian granulosa cells, at various stages of follicular development.

CELLS OF THE FOLLICULAR GRANULOSA AND THE INTERLEUKINS

The interleukins were first identified as hematopoietic growth factors present in the conditioned medium derived from discrete cellular populations (Dexter T.M., 1984). Currently, this family of factors includes members ranging from Interleukin-1 (IL-1) to interleukin-27 (IL-27), the most recent addition (Planz et al., 2002). As a group, the interleukins are most frequently described as modulators of immune cell functions.

Over the course of the last decade or so, a number of the interleukins have been reported in association with the cells and/or the follicular fluid isolated from ovarian follicles, including IL-1 (Baranao et al., 1995), IL-2 (Orvieto et al., 1997), IL-4 (Piccinni et al., 2001), IL-6 (Gorospe and Spangelo, 1993), IL-8 (Runesson et al., 2000), IL-10 (Calogero et al., 1998), IL-11 (Branisteanu et al., 1997), and IL-12 (Coskun et al., 1998). In some cases, however, the source(s) of these cytokines is either unknown or suspected to be immune cells resident within or just outside the follicle. Why are several interleukin molecules present within the follicle and why are some of these lymphokines synthesized specifically by the cells of the follicular granulosa?

The focus of the present discussion will be restricted to those interleukin molecules, and where possible their respective cellular receptors, believed to be synthesized by the cells of the follicular granulosa themselves, either during specific stages of follicular development and/or during formation of the corpus luteum, namely IL-1, IL-2, IL-6 and IL-8. A fairly indepth analysis of the signaling pathways, cellular occurrence and reported functions of each of these lymphokines is presented. This is done to establish the basis for suggesting that each of these molecules is a direct effector of granulosa cell function(s), in addition to any role(s) they might play in modulating immune cell function(s) either inside or outside the bounds of the follicle. However, as in the case of endothelial cells, it should be recognized that the boundaries between immune and nonimmune function(s) might sometimes be obscure and difficult to discern in a complex, carefully integrated biological system - the ovarian follicle.

IL-1

Current understanding of the IL-1 activation pathway suggests it operates principally through the utilization of 3 ligand forms, IL-1 α , IL-1 β and IL-1 ra (IL-1 receptor antagonist), and two receptor forms, IL-1 type I and type II receptors. IL-1 is synthesized by a wide variety of cells including monocytes/macrophages (Lasfargues et al., 1987), B cells (Matsushima et al., 1985), T cells (Acres et al., 1987), neutrophils (Tiku et al., 1986), astrocytes (Martin et al., 1992), fibroblasts (Le et al., 1987), epithelial (Cohen-Kaminsky and Berrih-Aknin, 1988), endothelial cells (Miossec et al., 1986) and follicular granulosa cells (Simon et al., 1994; Piquette et al., 1994). With respect to the cells of the ovarian follicle, among the more obviously relevant activities attributed to IL-1 signaling are: a) the stimulation of collagenase, stromelysin, prostaglandin, IL-6 and IL-8 synthesis by fibroblasts (Ridley et al., 1997), b) the induction of adhesion molecule expression in endothelial cells (Hawrylowicz et al., 1991), c) modulation of reparative functions following tissue injury (Wilson et al., 1996) and an ability to accelerate wound healing (Boisjoly et al., 1993), d) the induction of IL-2 receptor and cytokine production (Lowenthal et al., 1986), e) the inhibition of

gonadotropin-supported androgen production in the ovary and the inhibition of the induction of LH/hCG receptors in response to FSH (Hurwitz et al., 1991; Kasson and Gorospe, 1989), and f) an intermediary role in the ovulatory process of the ovarian follicle (Kol et al., 1999).

Each of the three IL-1 ligands, IL-1 α (Simon et al., 1994), IL-1 β (Piquette et al., 1994; Baranao et al., 1995), IL-1 ra (Hurwitz et al., 1992; Kol et al., 1999), and the two IL-1 receptor forms, IL-1 type I (Piquette et al., 1994; Simon et al., 1994) and type II receptors (Kol et al., 1999), have been reported to be synthesized by the cells of the follicular granulosa.

IL-2

Presently, the IL-2 signaling pathway is believed to involve several receptor species each with variable binding affinities for the primary ligand, IL-2. The high affinity IL-2 receptor (IL-2R) is a heterotrimeric molecular species containing associated IL-2R α (Tac, p55), IL-2R β (p70) and IL-2R γ (p64) binding subunits (Hermann and Diamantstein, 1988). The IL-2R α molecule by itself represents the low affinity receptor (Sabe et al., 1984). The intermediate affinity IL-2 receptor is a heterodimeric molecule containing IL-2R β and IL-2R γ binding subunits (Voss et al., 1992). Soluble IL-2 receptors (sIL-2R) involving all three IL-2R binding subunits (α , β and γ) have now been identified (Dummer et al., 1996). sIL-2Rs have been postulated to play regulatory roles by modulating the amount of free ligand available for IL-2 pathway signaling (Rubin et al., 1985).

Though, initially, the IL-2 signaling pathway was believed to be operative only in cellular subpopulations of the immune system, in particular T cells, B cells, natural killer cells and lymphokine-activated killer cells, more recently additional, seemingly nonimmune-associated, roles for this cytokine have been reported in several other cellular systems. For example, there are indications that IL-2 supports survival and neurite extension in cultured neurons, cell proliferation and maturation in oligodendrocytes, and it effects hypothalamic-pituitary function and electrocorticogram spectrum changes (Jiang et al., 1998). IL-2 supports cell proliferation and modulates ion secretion in cultured human small intestinal enterocytes (O'Loughlin et al., 2001). In rat intestinal epithelial cells grown in vitro, IL-2 has been shown to effect cell migration (Dignass and Podolsky, 1996) and to initially stimulate then subsequently inhibit cell division (Ciacci et al., 1993). IL-2 has also been reported to support proliferation in human microvascular endothelial cells (Hicks et al., 1989) and to alter the expression of adhesion molecules in human embryonic fibroblasts (Plaissance et al., 1994). Receptors for interleukin-2 have been identified on human endothelial (Hicks et al., 1991) and human fibroblast cells (Plaissance et al., 1992), as well as other types of nonimmune cells. Perhaps of particular relevance to the ovarian follicle, IL-2 treatment has been associated with loss of capillary integrity, macromolecule

leakage, and the induction of vascular leak syndrome (VLS), (Baluna and Vitetta, 1997).

It has been shown that cultured human granulosa cells produce IL-2 (Orvieto et al., 1997) and both IL-2 and its soluble receptors, sIL-2R, have been detected in human follicular fluids (Barak et al., 1992). In addition, IL-2 treatment has been shown to significantly inhibit progesterone production in hCG-stimulated human granulosa-luteal cells grown in vitro (Wang et al., 1991), thus providing preliminary circumstantial evidence for the existence at least one form of the IL-2R on the cells of the follicular granulosa. The correlation between IL-2 therapy and VLS has led several investigators to examine the role of IL-2 during ovarian hyperstimulation syndrome (OHSS). To date, however, there is no solid evidence supporting the proposition that IL-2 plays a direct, meaningful role in the etiology of OHSS (Geva et al., 1997; Aboulghar et al., 1999; Artini et al., 2002).

The gamma subunit of the IL-2R (IL-2R γ_c) has been reported to be shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (Sugamura et al., 1996; Asao et al., 2001). This raises the possibility that additional members of this receptor family may also be present on the cells of the follicular granulosa. Presently, receptors for IL-2 (Hicks et al., 1991), IL-4 (Palmer-Crocker and Pober, 1995) and IL-15 (Angiolillo et al., 1997) have been identified on endothelial cells.

IL-6

The IL-6 signaling pathway operates on the basis of complexes formed through the interaction of at least three molecular species: a) the IL-6 ligand, b) the soluble IL-6 receptor alpha protein (sIL-6R α), and c) the signal transducing gp130 protein. Together, these proteins assemble to yield a heterohexameric, high affinity, IL-6R receptor complex containing two IL-6, two IL-6R α and two gp130 molecules (Ward et al., 1994; Paonessa et al., 1995). IL-6 must first interact with the sIL-6R α protein, which binds the ligand with low affinity (Yamasaki et al., 1988), in order to bind and activate the gp130 protein. The binding of the IL-6/IL-6R α complex to the gp130 protein results in high affinity IL-6 binding (Hibi et al., 1990) and activation of the appropriate signal transduction pathways (Chow et al., 2001), something the IL-6/IL-6R α complex is incapable of doing on its own.

The IL-6 ligand is known to be produced by a wide variety of cells including monocytes/macrophage (Kotloff et al., 1990), astrocytes (Aloisi et al., 1992), fibroblasts (Yang et al., 1988), keratinocytes (Partridge et al., 1991), bone marrow stromal cells (Gimble et al., 1989), epithelial cells (Sakamoto et al., 1991), T (Zubiaga et al., 1990) and B cells (Rieckmann et al., 1991), endothelial cells (Podor et al., 1989), and ovarian follicular granulosa cells (Machelon et al., 1994). IL-6 is believed to be an important molecule during the induction of acute inflammatory responses and it has also

been shown to function as a differentiation and/or growth factor for B cells (most effective in the presence of IL-1 (Vink et al., 1988)), T cells (Uyuttenhove et al., 1988), and osteoclasts (Rozen et al., 2000). It has been suggested that the IL-6 protein may indirectly influence angiogenic events by binding to and modulating expression from the VEGF gene (Cohen et al., 1996).

Human follicular granulosa cells not only produce the IL-6 ligand, in addition they have been shown to synthesize the other components of the IL-6 activating pathway, IL-6R and the gp 130 protein (Salmassi et al., 2001). During in vitro analysis of these cells, increasing doses of IL-6 decreased the production of estradiol in the presence or absence of follicle stimulating hormone (FSH) and decreased the production of progesterone in the presence of FSH (Salmassi et al., 2001). In ovarian granulosa cells isolated from the rat, IL-6 treatment was shown to inhibit the production of estradiol and the expression of cytochrome P450 aromatase (Tamura et al., 2000). In OHSS, elevated IL-6 levels have been detected in the serum (Artini et al., 2002), ascitic fluid (Aboulghar et al., 1999) and follicular fluid (Geva et al., 1997) of affected women, in apparent agreement with the effect of IL-6 treatment on the permeability of endothelial cells grown in vitro (Maruo et al., 1992).

Finally, several different cytokines signal by way of an interaction of their ligand-specific alpha chains with the signal transducing gp 130 protein, a protein now known to be present on ovarian granulosa cells. This IL-6 receptor family of factors includes Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), Interleukin-11 and Cardiotrophin (CT-1), (Taga and Kishimoto, 1997; Bravo and Heath, 2000). Among these molecules, LIF (Coskun et al., 1998; Arici et al., 1997), CNTF (Watanobe and Habu, 2001), IL-11 (Branisteanu et al., 1997) and CT-1 (Pennica et al., 1996) have already been identified within the ovary (CT-1), the follicle (LIF, IL-11) or have been shown capable of exerting an effect on reproductive function (CNTF).

Noteably, all known members of the IL-6 receptor family of ligands - IL-6 (Maruo et al., 1992), LIF (Kimura et al., 2002), OSM (Vasse et al., 1999), CNTF (Koh, 2002), IL-11 (Mahboubi et al., 2000) and CT-1 (Jougasaki et al., 2002) - have been shown to be effector molecules of endothelial cell function(s).

IL-8

Currently, two IL-8 receptors have been identified in the IL-8 signaling pathway, CXC receptor 1 (CXCR1, formerly IL-8R-A) and CXC receptor 2 (CXCR2, formerly IL-8R-B), these two molecules share approximately 78% sequence homology (Lee et al., 1992). The "CXC" in the receptor designation refers to the fact that these are receptors for CXC chemokines, small activating molecules in which the first two cysteines are separated by a single

amino acid (Baggiolini et al., 1997). While IL-8 binds both CXCR1 and CXCR2 with high affinity, nearly all other known IL-8 receptor ligands (ELR⁺-CXC ligands (ELR represents a common Glu-Leu-Arg motif preceding the first cysteine in members of the group)) bind with high affinity only to CXCR2 (Ahuja and Murphy, 1996). In CXCR1 and CXCR2, signal transduction occurs as a result of coupling with cytoplasmic G proteins (Barnett et al., 1993), following successful interaction with a range of potential activating ligands. These ligand molecules include IL-8, growth-related oncogene (GRO) α , GRO β , GRO γ , neutrophil-activating peptide-2 (NAP-2), epithelial cell-derived neutrophil activating peptide -78 (ENA-78), and granulocyte chemoattractant protein-2 (GCP-2), (Baggiolini et al., 1997). Among these chemokines, only IL-8 and GCP-2 are effective ligands for CXCR1 (Wolf et al., 1998). All ELR⁺-CXC ligands attract and activate neutrophils (Baggiolini et al., 1997).

IL-8 has been shown to be produced by epithelial cells (Azghani et al., 2002), fibroblasts (Sempowski et al., 1998), immune cells, including T cells (Harris et al., 2002) and macrophages (Fu et al., 2002), endothelial (Hippenstiel et al., 2000), and follicular granulosa cells (Runesson et al., 1996; Chang et al., 1998), among others. CXCR2 has been identified on a variety of cells including epithelial cells in psoriasis (Schulz et al., 1993), melanocytes and fibroblast (Moser et al., 1993), and smooth muscle cells during wound repair (Nanney et al., 1995). The presence of both CXCR1 and CXCR2 has been reported on dendritic cells (Sozzani et al., 1997), immune cells, including neutrophils, monocytes, NK cells and some lymphocytes (Chuntharapai et al., 1994; Morohashi et al., 1995), and endothelial cells (Schraufstatter et al., 2001). At present there are no literature reports regarding the expression of CXCR1 or CXCR2 by the cells of the follicular granulosa.

In line with its roles as a major attractant and activator of leukocytes, IL-8 is an important epithelial and endothelial cell inflammatory mediator capable of inducing neutrophil chemotaxis and transmigration (Ina et al., 1997; Huber et al., 1991). IL-8 has been reported to support the proliferation of keratinocytes in vitro and to enhance wound healing in vivo, in part as a consequence of its effects on cell proliferation (Tuschil et al., 1992; Rennekampff et al., 2000). Also in epithelial cells, IL-8 has been shown to stimulate the migration of colonic epithelial cells in an in vitro wound-healing model (Wilson et al., 1999). In endothelial cells, IL-8 has been implicated in migration (Szekanecz et al., 1994), cell permeability (Biffl et al., 1995), proliferation and survival (Aihua et al., 2002). In addition, IL-8 has been shown to promote angiogenesis (Hu et al., 1993; Koch et al., 1992).

Why are IL-1, IL-2, IL-6 and IL-8 present within the ovarian follicle? In particular, why are these cytokines produced by the cells of the follicular granulosa? Some have suggested that ovulation is akin to an inflammatory response or process (Espey, 1994). If this is so, which cells are active

participants in that response or process? Are granulosa cells among them? In mesothelial cells, hyaluronan fragments have been shown to induce the expression of monocyte chemoattractant protein 1 (MCP-1) and IL-8 (Haslinger et al., 2001). Does enzymatic digestion of the high molecular weight hyaluronan associated with the follicle, perhaps around the time of follicular rupture, effect the expression of MCP-1 and IL-8 by the cells of the follicular granulosa? Almost invariably, as each new cytokine is identified in association with the ovarian follicle the reflex conclusion is that its role must be to effect the function of immune cells associated with the follicle. Yet, in nearly each instance, as more information becomes available, it is determined, as in the case of endothelial cells, that the particular cytokine in question is produced by and is an effector molecule of the cells of the follicular granulosa, in addition to any role(s) it may have with respect to the associated immune cells of the follicle. In the body in general, endothelial cells, and the blood vessels they form, are frequently conduits for immune cell activity, the rallying and launching points for immune cell function. Once ovulation occurs, how are the immune cells directed, guided, their activities altered and modified to achieve the desired objective? Is it possible that the direction, guidance, alteration and modification of immune cell function is one of the important charges allotted to those granulosa cells remaining behind with the ovulated follicle? Might one of the functions of the various cytokines present within the follicle be to direct and integrate the behaviors and activities of both granulosa and immune cells in order to efficiently and effectively transform the ruptured follicle into a fully functional and integrated corpus luteum?

CELLS OF THE FOLLICULAR GRANULOSA, ANGIOGENIC GROWTH FACTORS AND THEIR RECEPTORS

Ovarian follicular granulosa cells have been reported to synthesize and secrete a variety of angiogenic factors in addition to the cytokine molecules previously discussed. These include bFGF (Di Blasio et al., 1993), endothelin-1 (Magini et al., 1996), leptin (Cioffi et al., 1997), TGF β 1 (Mulheron et al., 1992), angiogenin (Koga et al., 2000), angiopoietin-1 (Hazzard et al., 1999), angiopoietin-2 (Maisonneuve et al., 1997), and VEGF (Van Blerkom et al., 1997). These same cells have also been shown to express receptors for bFGF (Di Blasio et al., 1993), Endothelin-1 (Gentili et al., 2001), leptin (Cioffi et al., 1997), TGF β 1 (Roy and Kole, 1998), angiopoietin-1, angiopoietin-2 (Antczak and Van Blerkom, 2000), and VEGF (VEGF-R1 (Flt-1): Otani et al., 1999; Antczak and Van Blerkom, 2000). Why do the cells of the follicular granulosa synthesize and secrete these angiogenic factors? Perhaps more importantly, why do the granulosa cells express cellular receptors for these angiogenic factors?

ENDOTHELIN-1 AND ENDOTHELIN RECEPTORS A AND B

The synthesis of endothelin-1 (ET-1), a 21 amino acid vasoactive peptide, has been identified in smooth muscle, epithelial, fibroblast (Saenz de Tejada et al., 1992), neural (Giard et al., 1989), amnion (Casey et al., 1991), endothelial (Takahashi et al., 1989) and follicular granulosa cells (Magini et al., 1996). Two basic endothelin-1 cellular receptor forms are currently known to exist. These receptors signal by coupling to G proteins (Arai et al., 1990; Sakurai et al., 1990) and their standard designations are ET_A and ET_B. ET_A receptors bind endothelin-1 and endothelin-2 molecules with high affinity (ET-1>ET-2), (Arai et al., 1990). ET_B receptors bind all three of the known endothelin ligands (ET-1, -2 and -3) with similar affinities, (Sakurai et al., 1990). Vascular smooth muscle cells are believed to interact with ET-1 through the expression of ET_A and ET_B receptors, both of which have been shown capable of mediating contractions in these cells (Sumner et al., 1992). Endothelial cells, with the exception of capillary endothelial cells which express both ET_A and ET_B, are believed to interact with ET-1 molecules primarily through ET_B receptors (arterial and venous endothelial cells), (Wendel-Wellner et al., 2002), these receptors are thought to regulate postcapillary vasodilation. Interactions between the ET_B receptors on limbal vessels and the ligands ET-1 and ET-3 have been implicated in the induction of angiogenic responses in the rat corneal angiogenesis assay (Bek and McMillen, 2000).

As is true for vascular smooth muscle and capillary endothelial cells, human granulosa cells have been reported to express message for both ET_A and ET_B receptors, though ET_B message was considerably less abundant and the respective protein was not detectable by immunocytochemical analysis (Gentili et al., 2001). Further, in human granulosa-lutein cells it has been suggested that endothelins, acting primarily through the ET_A receptor, are capable of blocking cyclic AMP-dependent FSH-mediated functions (Furger et al., 1995), of stimulating cell proliferation, and attenuating basal progesterone secretion (Kamada et al., 1995). While in the case of rat granulosa cells, endothelins reportedly inhibit estrogen and cAMP production (Calogero et al., 1998), in addition to progesterone synthesis (Tedeschi et al., 1994). Also in rat granulosa cells, FSH treatment induced a dose-dependent increase in ET-1 binding while LH treatment induced a dose-dependent decrease in ET-1 binding (Otani et al., 1996).

Why are ET-1 molecules and at least two functional receptor forms expressed by the cells of the follicular granulosa?

ANGIOPOETIN-1, ANGIOPOETIN-2 AND TEK AND TIE-1 RECEPTORS

The term “angiopoietin” was chosen to highlight the role of the first identified member of the ligand family (angiopoietin-1) in angiogenesis as well as its potential role in hematopoiesis (Davis et al., 1996). In general, the angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) molecules exert opposing effects on angiogenesis through their interactions with a common tyrosine kinase cellular receptor designated Tie-2 or Tek (Maisonpierre et al., 1997). The Tie designation is an acronym for tyrosine kinase with immunoglobulin and EGF homology domains (Partanen et al., 1992). Expression of the Ang-1 and/or Ang-2 molecules has been identified in, astrocytes (Ang-1, Acker et al., 2001), pericytes (Ang-1, Sundberg et al., 2002), smooth muscle (Maisonpierre et al., 1997; Mandriota and Pepper, 1998), hematopoietic (Ang-1, Muller et al., 2002), endothelial (Ang-2, Huang et al., 2002), and follicular granulosa cells (Ang-2, Maisonpierre et al., 1997; Ang1 and Ang-2, Hazzard et al., 1999). Expression of the Tek receptor has been identified in hematopoietic (where it is believed to be a marker for stem cells (Yuasa et al., 2002)), endothelial (Dumont et al., 1994), and follicular granulosa cells (Antczak and Van Blerkom, 2000; Wulff et al., 2001).

In endothelial cells, it is believed that the angiopoietins and the Tie-2 receptor play important roles during angiogenic outgrowth, and during the remodeling and maturation of primitive vessels rather than participating in the early vasculogenic phases of vessel development (Maisonpierre et al., 1997). During these activities the angiopoietins and the Tek receptor are believed to mediate signaling between the endothelial cells and surrounding mesenchymal cells to establish contacts and interactions which maintain and stabilize more mature blood vessels (Maisonpierre et al., 1997). In addition, there are reports that Ang-1 may protect endothelial cells from apoptosis (Kwak et al., 1999), and that the expression of Ang-2 may be effected by hypoxia (Mandriota and Pepper, 1998). Knockout mice devoid of functional Ang-1 (Suri et al., 1996), the Tek receptor (Dumont et al., 1994) or overexpressing Ang-2 (Maisonpierre et al., 1997) display similar vascular abnormalities and die in utero during embryonic development, underscoring the importance of each of these molecules during vascular development.

During message analysis of granulosa cells derived from the unstimulated, macaque, ovarian follicles the presence of both Ang-1 and Ang-2 mRNAs were detected. In this model system, hormonal stimulation (hCG) was reported to modulate Ang-1 mRNA levels (initial decrease then large increase) but had no effect on Ang-2 mRNA levels, while the levels of both mRNAs were reduced by steroid ablation in a time specific fashion and only Ang-1 levels could be restored by subsequent progestin treatment (Hazzard et al., 1999).

The production of the Ang-1 and Ang-2 proteins by the cells of the follicular granulosa provided one of the bases to test the hypothesis that follicular granulosa cells represent an endothelial-like cell population. Specifically, because the angiopoietins were produced by a population of cells residing in a closed system, the follicle, would these same cells, the follicular granulosa, also express the Tek receptor? Further, would the granulosa cells express a second, structurally related, tyrosine kinase receptor molecule, Tie-1, also shown to be largely restricted to cells of the endothelial and hematopoietic lineages (Partanen et al., 1992)? The Tie-1 protein has been reported to be important for the survival and integrity of vascular endothelial cells and vessels they create (Puri et al., 1995). Representative results demonstrating expression of both the Tie-1 and Tek proteins in ovulated, murine, cumulus-oocyte-complexes are shown in figures 1.2A and 2B, respectively. Similar results obtained following the analysis of the expression of the Tie-1 and Tek proteins in a) cultured, preovulatory, human, follicular granulosa cells, b) isolated, murine, primordial follicles, and c) paraffin sections of murine ovaries have been published previously (Antczak and Van Blerkom, 2000). In murine COCs, many cells of the corona radiata (directly contacting the zona pellucida) and the cumulus oophorus stained brightly for the Tie-1 (2A) and Tek (2B) proteins. Results from similar murine COCs analyzed to determine levels of nonspecific antibody interactions and background fluorescence are shown in figure 1.2D, for comparison. Only low-level, nonspecific, background levels of fluorescence were associated with antibody control samples.

The results described here and elsewhere regarding the expression of the Tek protein by the cells of the follicular granulosa have been corroborated by other investigators following the detection of Tek (Tie-2) mRNA and protein in the granulosa cells of early follicular stages in the marmoset monkey (Wulff et al., 2001).

Why do the cells of the follicular granulosa express the Tie-1 and Tek receptor tyrosine kinases and the molecules angiopoietin-1 and angiopoietin-2?

VEGF RECEPTORS

Of all the angiogenic factors produced by the cells of the follicular granulosa none have received more attention than the VEGF ligands. Follicular granulosa cells have been reported to express multiple VEGF-related molecules including VEGF-121, -145, -165, VEGF-B and VEGF-C (Laitinen et al., 1997). These ligands are produced by a wide variety of cells in addition to ovarian granulosa cells. In the present discussion, the important issue is not the source but rather the possible target(s) for the various VEGF species produced by the granulosa cells. Therefore, here, the expression of receptors for the VEGF ligands will be the primary focus of attention.

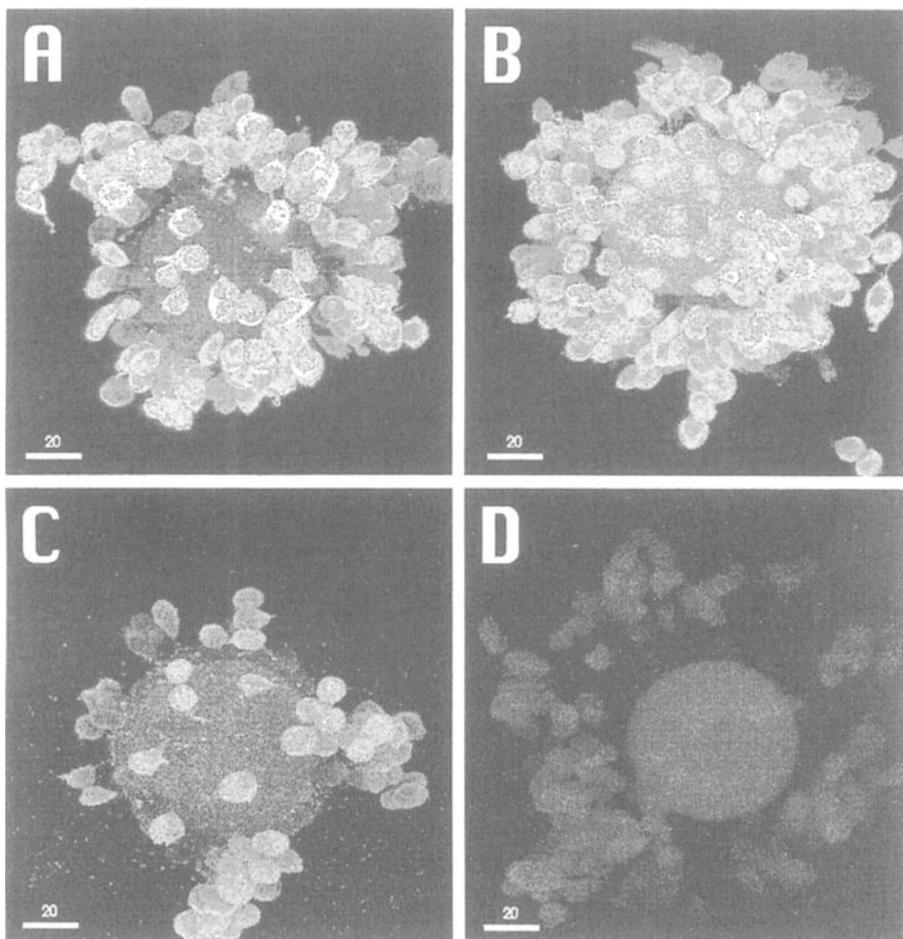


FIGURE 1.2. Results following indirect immunofluorescent analysis of the expression of the Tie (A), Tek (B) and Flt-1 tyrosine kinase cellular receptors in freshly isolated, ovulated, murine, cumulus-oocyte-complexes Representative antibody control results showing levels of nonspecific antibody interactions and background fluorescence are show in D. See text for additional details.

Several receptor molecules for the VEGF ligand have been identified to date, included among them are the fms-like tyrosine kinase, Flt-1 (VEGF-R1), (de Vries et al., 1992), the kinase insert domain-containing receptor, KDR (Flk-1 in mouse (VEGF-R2), (Terman et al., 1991), neuropilin-1 (NP-1), (Soker et al., 1998) and neuropilin-2, (NP-2), (Gluzman-Poltorak et al., 2000).

Though original thought to be expressed exclusively in endothelial cells, expression of the Flt-1 and KDR receptors has now been identified in other cell types including, for Flt-1, smooth muscle (Wang and Keiser, 1998),

osteoblast (Mayr-Wohlfart et al., 2002), monocytes-macrophage (Sawano et al., 2001), spermatozoa (Obermair et al., 1999), trophoblast (Shore et al., 1997), pancreatic islet cells (Christofori et al., 1995), and follicular granulosa cells (Otani et al., 1999); for KDR, spermatozoa (Obermair et al., 1999), osteoblast (Mayr-Wohlfart et al., 2002), pancreatic islet cells (Christofori et al., 1995), and granulosa lutein cells (Endo et al., 2001).

The neuropilins were originally identified in neural tissues (Takagi et al., 1987; Kolodkin et al., 1997) but have subsequently been described in other tissues. For example, neuropilin-1 has been reported in association with osteoblasts (Mayr-Wohlfart et al., 2002), glomerular epithelium (Harper et al., 2001), uterine glandular epithelium (Pavelock et al., 2001), and endothelial cells (Soker et al., 1998). Neuropilin-2 has been identified in amnion cells (Marvin et al., 2002), neuroendocrine cells of the digestive tract (Cohen et al., 2001) pancreatic islet cells (Cohen et al., 2002), uterine glandular epithelium (Pavelock et al., 2001), osteoblasts (Mayr-Wohlfart et al., 2002), and endothelial cells (Gluzman-Poltorak et al., 2000). The neuropilin receptors are particularly interesting because they are known to be receptors for class-III semaphorin/collapsin guidance molecules (Sema A- F), (Kolodkin et al., 1997) in addition to being receptors for specific VEGF isoforms. Class-III semaphorins/collapsins typically induce repulsion of growth cones on NP-1 and NP-2 expressing neural cells (Lou et al., 1993). Of particular relevance to the present discussion, class-III semaphorins/collapsins have recently been reported to inhibit endothelial cell motility and angiogenesis (Miao et al., 1999).

In endothelial cells, VEGF and its receptors are believed to be the prime regulators of vasculogenesis and angiogenesis, in contrast to the effects of the angiopoietins and the Tie-1 and Tek (Tie-2) receptors. For example, Flt-1 (VEGF-R1) knockout mice form endothelial cells but these endothelial cells are unable to assemble into a functional vasculature, the embryos die in utero (Fong et al., 1995). In KDR/flk-1 (VEGF-R2) knockout mice, endothelial cell differentiation does not occur and therefore blood vessels do not form, the embryos die in utero (Shalaby et al., 1995). Mice devoid of functional NP-1 and NP-2 receptors develop avascular yolk sacs, the effected embryos die in utero (Takashima et al., 2002). Mice lacking either the NP-1 or NP-2 receptor alone have been reported to have a greatly reduced yolk sac vasculature, disorganized blood vessels and growth-retarded embryos which die in utero (Takashima et al., 2002).

If the cells of the follicular granulosa truly represent a specialized endothelial-like cell population, they would be expected to express cellular receptors for VEGF, a molecule which would be of fundamental importance to the expression of their endothelial character. They would be expected to exhibit cellular receptors that would enable them to interact with at least a portion of the VEGF molecules they synthesize and secrete. Initially, this hypothesis was tested by examining the expression of the Flt-1 protein in

human and murine follicular granulosa cells. Flt-1 was chosen because like VEGF (Shweiki et al., 1992), and unlike KDR/Flk-1, its expression is upregulated by hypoxia (Gerber et al., 1997), a condition likely to exist during maturation of the ovarian follicle. Shown in figure 1.2C are results obtained following immunofluorescent analysis of the Flt-1 protein in ovulated murine COCs. Immunofluorescent and Western analysis of Flt-1 expression by human follicular granulosa cells has been published previously (Antczak and Van Blerkom, 2000). Cells of the corona radiata (in direct contact with the zona pellucida) and the cumulus oophorus stained positively for the Flt-1 protein (Figure 1.2C). Corresponding specimens examined for nonspecific antibody interactions and levels of background staining were associated with signals of low fluorescence intensity (Figure 1.2D). These Flt-1 results support a previously published report regarding the expression of the Flt-1 protein by human follicular granulosa cells (Otani et al., 1999).

In an effort to continue to test the possible link between endothelial cells and granulosa cells and in view of the strategic role played by VEGF during the expression of many endothelial cell behaviors and functions, human and murine granulosa cells were recently examined further for the expression of additional VEGF receptor species. Preliminary results suggest that, in addition to the Flt-1 receptor, human and murine follicular granulosa cells also express the NP-1 and NP-2 VEGF receptors (unpublished finding). If these preliminary findings prove correct, the expression of 4 unique VEGF receptor molecules (Flt-1, KDR, NP-1 and NP-2) will be associated with either follicular or luteal granulosa cells.

Why do cells of the follicular granulosa express at least 5 VEGF or VEGF-related molecular species (VEGF-121, -145, -165, VEGF-B and VEGF-C)? Why would the cells of the follicular granulosa express, potentially, at least three different VEGF receptor molecules (Flt-1, Np-1, and NP-2) if they were not at least one of the primary targets for the various VEGF-related molecules they synthesize and secrete? If the cells of the follicular granulosa are targets for the multiple VEGF signaling pathways they express, what types of cellular activities do these pathways influence and/or control?

CELLS OF THE FOLLICULAR GRANULOSA AND THE BIOACTIVE MOLECULE HYALURONAN

Hyaluronic acid or hyaluronan (HA) is present in such abundance during ovulation that its physical presence can be easily detected, with the assistance of a simple dissecting microscope, as one of the principle matrix components supporting the cumulus cells in the cumulus-oocyte-complex (COC). Yet for all its abundance, relatively little is known about the functional significance of this molecule in the follicle and in reproductive biology in general. Why is hyaluronan present within the follicle and why is so much of it present? Some would argue that the role of hyaluronan is simply to increase the physical size

of the mass associated with the ovulated oocyte in order to facilitate its movement through the oviduct. While this view, this dogma, may be partially correct, it may be time to remove an association with the word "simply" and carefully consider some broader and more fundamental possibilities.

Hyaluronan is a disaccharide molecule consisting of D-glucuronic and N-acetyl-D-glucosamine. It was first identified in 1934 as a component of the vitreous of the eye. The name, hyaluronic acid/hyaluronan originates from a combination of hyaloid, the Greek word for vitreous (the source), and hexauronic acid (D-glucuronic acid), (the identified component), (Meyer and Palmer, 1934). Typically, the molecule is assembled into long linear polymers via the actions of the membrane-bound hyaluronan synthase enzymes located at the cell surface. Currently, it is believed that the growing HA chain is extruded through the cell membrane into the extracellular space as biosynthesis of the molecule continues (Weigel et al., 1997). Oligomeric complexes of hyaluronan can exceed 30,000 units and a molecular mass of ten million daltons (Weigel et al., 1997). Once considered simply a widely distributed structural component of extracellular matrices, a new awareness is developing with respect to hyaluronan and its possible bioactive properties. Included among these properties are morphogenesis, where HA knockout mice died during early embryonic development (E9.5-10) with severe cardiac and vascular abnormalities (Camenisch et al., 2000), proliferation (Greco et al., 1998), differentiation (Nilsson et al., 2002), cell migration (Toole et al., 1997), wound healing (Chung et al., 1999), angiogenesis (Montesano et al., 1996), and the promotion of certain types of cancer (Hiltunen et al., 2002; Lokeshwar et al., 2001)

HA is synthesized and secreted by fibroblasts (Ellis et al., 1997), epithelial (keratinocytes (Akiyama et al., 1994)), smooth muscle (Merrilees et al., 1990), endothelial (Mohamadzadeh et al., 1998 (microvascular endothelial cells)) and granulosa (Tirone et al., 1997) cells, among others. HA can be found in virtually all the tissues of the body.

A relatively large number of hyaluronan receptors have been identified to date. Four, extracellular, cell associated, HA receptors will be briefly discussed here: 1) CD44, 2) the receptor for HA-mediated motility (RHAMM (CD168)), 3) the HA receptor for endocytosis (HARE (liver sinusoidal endothelial cells)), and 4) the intercellular adhesion molecule-1 (ICAM-1 (CD-54)).

In view of the wide tissue distribution of hyaluronan, it is not surprising that CD44 is expressed ubiquitously, including human sperm (Bains et al., 2002). Many different isoforms of CD44 (Borland et al., 1998), as well as a soluble form of the receptor (Yang and Binns, 1993), have been identified. Interest in the alternate forms of the CD44 receptor has been especially keen in the field of cancer research, in part because of their potential use as diagnostic markers of the disease state (Hudson et al., 1996; Yokoyama and Yamaue, 2002).

RHAMM expression has been identified on a variety of cells including neurons, astrocytes and microglia (Nagy et al., 1995; Turley et al., 1994), smooth muscle (Savani et al., 1995), lymphocytes and thymocytes (Pilarski et al., 1994) human sperm (Kornovski et al., 1994), tumor cells (Kong et al., 2003), endothelial (Lokeshwar and Selzer, 2000), and granulosa (Stojkovic et al., 2003) cells. Soluble forms of RHAMM are known to exist and are biologically active (Mohapatra et al., 1996). In the majority of cases, as its acronym implies, RHAMM has been found to be associated with, among other things, cell motility. This does not preclude a similar capacity by other HA receptors.

To date, HARE has been reported in association with liver sinusoidal endothelial cells (Weigel et al., 2002), venous sinuses of red pulp in the spleen and the medullary sinuses of lymph nodes (Zhou et al., 2000). The HARE receptor internalizes HA and chondroitin sulphate and is associated with an internal pathway that results in the complete destruction of the both molecules. This process is important in maintaining relatively low systemic levels of these molecules despite the nearly constant degradation and release of HA and chondroitin sulphate from the various tissues of the body (Zhou et al., 2002).

ICAM-1 is expressed on vascular smooth muscle cells in atherosclerotic lesions (Printseva et al., 1992), epithelial, fibroblast, macrophage, mitogen-stimulated T lymphocyte blasts, leukocytes, endothelial (Dustin et al., 1986), and granulosa cells (Vigano et al., 1997). ICAM-1 is a ligand for lymphocyte-function associated (LFA) antigens, molecules expressed on T cells, B cells, granulocytes and other leukocytes (Simmons et al., 1988), and also for the macrophage antigen-1 (Mac-1, (Diamond et al., 1990)) expressed on macrophage, neutrophils and other immune cells. ICAM-1 is a strategically important mediator of immune cell function. In targeted cells or tissues, for example endothelium, its expression is upregulated by inflammatory cytokines (i.e., IL-1 β and TNF α (tumor necrosis factor alpha (Mohamadzadeh et al., 1998)) resulting in the recruitment and localization of immune cell subpopulations and the modulation of their behaviors. Soluble forms of CD54 have been identified (Rothlein et al., 1991). These soluble forms have been shown to be present in human follicular fluid and secreted by ovarian granulosa cells (Vigano et al., 1998). Considerable efforts are currently underway to determine the significance and the diagnostic value of soluble ICAM-1 molecules in various diseases, including cancer (Maruo et al., 2002) and atherosclerosis (Hulthe et al., 2002), among a great many others.

Morphogenesis, the induction of cell proliferation and differentiation, the promotion of cell migration, angiogenesis and wound healing, the recruitment and modulation of immune cell function, these are some of the biological potentials currently attributed to hyaluronan via the actions of its cellular receptors, including CD44, RHAMM and ICAM-1. These biological

potentials are required during the formation and maintenance of the vascular system and for the proper function of its endothelial cells. These biological potentials are also required during the maturation of ovarian follicles and/or during formation of the corpus luteum. It is inconceivable, that the cells of the follicular granulosa synthesize copious amounts of a molecule capable of eliciting behaviors and activities so fundamental to their biology, that they express at least three cellular receptor forms (CD44, RHAMM and ICAM-1) which would allow them to successfully interact with that molecule, yet the only significant role assigned to that molecule is one of providing physical support and bulk. Using parallels to endothelial cell biology for guidance, there may be considerably more to the role of hyaluronan in the follicle and during formation of the corpus luteum than previously believed.

In the endothelial cells of small blood vessels, the primary site of HA production by endothelial cells (Mohamadzadeh et al., 1998), Interleukin 15 has been shown to induce the expression of hyaluronan and support the extravasation of activated T cells from the blood via the interaction of the CD44 receptors on the lymphocytes and the HA ligand on the endothelial cells (Estess et al., 1999). Though there are currently no reports regarding the presence of IL-15 within the follicle, IL-1 β and TNF α also induce the expression of hyaluronic acid in endothelial cells (Mohamadzadeh et al., 1998) and each of these molecules has been shown to be present within the follicle. In the follicle, it has been suggested that the secretion of TGF β and TNF α may be regulated by gonadotropins (Loret De Mola et al., 1998) and hyaluronan synthesis has been reported to begin shortly after the LH (leutinizing hormone) surge. Within the ovarian follicle there is at least one additional molecular species known to induce the synthesis of hyaluronan by the cells of the follicular granulosa, growth differentiation factor-9 (GDF-9). Perhaps underscoring the fundamental importance of HA to normal and successful follicular function, GDF-9 is produced by the oocyte itself to impact the production of hyaluronan by coresident follicular granulosa cells (Elvin et al., 1999). Why would the oocyte engage in the synthesis of GDF-9 to induce the production of HA by the cells of the follicular granulosa unless it had an important, vested interest in results of its efforts, though it is currently unclear where the focus of those interests might be?

Following ovulation, does the hyaluronan present within the follicle mediate the movement of immune cells into the ruptured follicle? In this regard it has been postulated that the presence of ICAM-1 molecules on the cells of the follicular granulosa may play a role in the accumulation, distribution and regulation of leukocytes in ovarian tissue (Vigano et al., 1997). Do hyaluronan, its receptors (soluble and membrane bound) and adhesion molecules expressed by granulosa cells and leukocytes support specific and directed interactions between follicle cells and immune cells, as in the case of endothelial cells? Do these interactions, if present, facilitate and

expedite the formation of the corpus luteum or the demise of ovulated follicles if fertilization fails to occur or developing embryos fail to implant?

In endothelial cells there is an unusual functional duality associated with hyaluronic acid and its effects on vasculogenesis and angiogenesis. High molecular weight forms of hyaluronan, ($\sim 10^6$ da), have been shown to inhibit blood vessel development (Deed et al., 1997) while low molecular weight forms, (4-25 disaccharide units), are potent inducers of this phenomenon (West et al., 1985). Blood vessel development during corpus luteum formation is an absolute necessity if the forming endocrine gland is to be fully integrated into the systemic circulation. A possible role for hyaluronan in containing or suppressing the expression of unique, morphodynamic, endothelial cell-like behaviors by the cells of the follicular granulosa during follicular development and an opposite, inductive, role for hyaluronan during the possible expression of mophodynamic, endothelial cell-like behaviors on the part of cells within the follicular granulosa, following ovulation and during formation of the corpus luteum, has been previously proposed (Antczak, 2001). Along these same lines, it has been proposed that the stores of hyaluronan within the intact follicle function as an “angiogenic shield” to prevent the entry of thecal endothelial cells into the interior of the follicle, in the form of angiogenic sprouts, until just prior to or following ovulation (Tempel et al., 2000). However, there are two relevant issues that impact the proposed target of this “angiogenic shield” hypothesis which were completely overlooked by the investigators. Namely, 1) the production of hyaluronan by the cells of the theca (Laurent et al., 1995) and 2) the inaccessibility of high MW, antiangiogenic, shielding, stores of hyaluronan within the follicle until breach of the follicular basal lamina is accomplished. In short, prior to ovulation, thecal endothelial cells would have to breach the basal lamina of the follicle in order to experience the effects of the angiogenic shield represented by the hyaluronan stores contained within. There is no evidence that endothelial cells do this until just prior to of following ovulation.

Earlier in this section a possible link was drawn between TNF α and the induction of hyaluronan in endothelial cells and cells of the follicular granulosa. There is at least one additional endothelial cell activity associated with the cytokine TNF α that bears mention at this point, in particular, the ability of TNF α to elicit angiogenic responses (Pandey et al., 1995). What is particular interesting with respect to the link between TNF α and angiogenesis is the belief that TNF α evokes its angiogenic responses, at least in part, through its effects on the ephrin signalling pathways (Pandey et al., 1995). Recent literature reports suggest that the ephrin ligands and receptors are of fundamental importance to endothelial cell functions and behaviors. If follicular granulosa cells truly represent a specialized type of endothelial-like cell, then it would be expected that the ephrins would also be an integral part of their signal transduction portfolio.

FOLLICULAR GRANULOSA CELLS AND THE EPHRINS

When discussing interleukin molecules within the follicle, the general tendency is to assume their exclusive role is to effect or modulate the functions of the immune cells associated with the follicle, whether internally or externally located, despite the presence of the appropriate receptors on the cells of the follicular granulosa. Similarly, when discussing angiogenic factors identified within the follicle, it is generally assumed that the primary function(s) of those factors is to influence vascular events occurring outside the follicle, despite the presence of the corresponding cellular receptors on the cells of the follicular granulosa. In order to more clearly define the purpose(s) for the synthesis of particular ligands and their cellular receptors by the cells of the follicular granulosa, it would be particularly useful to identify specific sets of matching ligands and cellular receptors where the activating molecules were not released free into the environment but required physical, cell-to-cell, contact for binding, receptor activation and signal transduction to occur. That is precisely the manner in which the Eph/ephrin signalling pathways are currently believed to operate, using juxtacrine ligand/receptor interactions (Orioli and Klein, 1997).

The terms Eph and ephrin result from the identification of the first member of the receptor family (EphA1), a sequence derived from an erythropoietin-producing hepatocellular carcinoma cell line (Hirai et al., 1987). Currently, the Eph ("Eph" is used to designate the corresponding receptor form (Eph nomenclature committee, 1997)) family, the largest known family of receptor tyrosine kinases, contains at least 14 members (EphA1-A8 and EphB1-B6). There are at least 8 different ephrin ("ephrin" is used to designate the corresponding ligand form (Eph nomenclature committee, 1997)) activating ligands (Gale and Yancopoulos, 1997). The ephrins are divided into two classes on the basis of their attachment to the plasma membrane. Class A ephrins (A1-A5) are secured to the cell membrane via glycosylphosphatidylinositol (GPI) linkages, they associate primarily with class A receptors (EphA). Class B ephrins (B1-B3) are attached to the cell surface using linkages containing both a transmembrane and conserved cytoplasmic domain, these molecules associate primarily with class B receptors (EphB), though EphA4 has been shown to bind ephrin B molecules (Flanagan and Vanderhaeghen, 1998). In vivo and in vitro studies suggest that the ephrin molecules must be presented in a clustered fashion in order to activate the appropriate receptors on the receiving cells. The degree of ephrin clustering may result in differential signaling from the interacting receptor(s) (Davis et al., 1994; Wang and Anderson, 1996; Stein et al., 1998).

The ephrin/Eph signaling pathways are unique among ligand/receptor interactions in that, upon binding, both the ligand(s) (reverse signaling) and the receptor(s) (forward signaling) are believed to transduce signals back into their respective cells, a process termed bidirectional signaling (Holland et al.,

1996). For the receptor molecules, forward signaling is accomplished in a manner typical for receptor tyrosine kinases, activation of the intracellular catalytic tyrosine kinase domain (Holland et al., 1998). For ephrinB molecules, reverse signalling is believed to occur as a result of phosphorylation of tyrosine residues on their cytoplasmic tails, following receptor binding (Bruckner et al., 1997). In the case of the ephrin A ligands, reverse signaling is believed to be mediated by an association with cytoplasmic tyrosine kinases (Src family) located on the intracellular surface of specialized domains (Davy et al., 1999), regions where the clustered ligands assemble.

Eph/ephrin molecules were first studied for their effects on the nervous system. They are believed to play important roles in axonal guidance, neural crest cell migration and neural patterning (Gale and Yancopoulos, 1997). It has been suggested that Eph/ephrin molecules are important in segmentation, cell migration and they may be involved in limb bud formation during embryonic development (Holder and Klein, 1999). Members of this signaling pathway have been described in many types of cells including epithelial (Coulthard et al., 2001), muscle (Feng et al., 2000), ovarian (Kozlosky et al., 1997), and endothelial cells (Mcbride and Ruiz, 1998). Presently, there is considerable interest in the Eph/ephrin family of molecules because of their possible functions during vasculogenesis (the formation of new blood vessels) and angiogenesis (the extension of preexisting blood vessels), (Wang et al., 1998; Adams et al., 1999).

To date, venous endothelial cells have been reported to express ephrinB1, EphB3 and EphB4 (Adams et al., 1999). Arterial endothelial cells have been reported to express ephrinB1, ephrinB2, in some cases EphB3 (Adams et al., 1999), as well as low levels of Eph B4, at least during early embryonic development (Gerety et al., 1999). Interestingly, during early vascular development, EphB4 was found to be expressed exclusively on cells of the vascular system (Gerety et al., 1999). EprinB2 (Wang et al., 1998) and EphB4 (Gerety et al., 1999) knockout mice reportedly died before day 11 of embryonic development, apparently as a result of similar cardiovascular defects. These findings suggest that EphB4 is an important receptor for ephrinB2 during early cardiovascular development (Gerety et al., 1999). EphrinA1-Ig chimeric proteins have been shown to be chemoattractive to endothelial cells in vitro, capable of inducing tube formation in human umbilical vein endothelial cells (HUVEC), and angiogenic in the rat corneal angiogenesis assay (Pandey et al., 1995; Daniel et al., 1996). EphrinB1-Ig and ephrinB2-Ig chimeric molecules have been shown to support capillary sprouting in vitro (Adams et al., 1999).

Another potentially important facet to the Eph/ephrin signalling molecules and their roles during vascular development and angiogenesis is the finding that adjacent non-endothelial cells can also express some of these molecules. For example, strong ephrinB2 and EphB2 signals were detected in

mesenchymal cells adjacent to endothelial expressing the complimentary receptors and ligands (Adams et al., 1999). This raises the possibility that fibroblast, muscle or other cell types in the immediate vicinity of endothelial cells may use a repertoire of ephrin ligands and/or receptors to modulate endothelial cell activities in order to achieve a desired outcome.

Based on the accumulated evidence that ephrin/Eph molecules are critical for specific types of endothelial cell function and the belief that follicular granulosa cells represent a specialized endothelial-like cell population, an examination of the expression of ephrinA1, ephrinB1, EphA1, EphB1 and EphA2 molecules in granulosa cells of both human and murine origin was conducted, by indirect immunofluorescence and Western analysis (data not shown).

Positive immunofluorescent signals were detected in both human and murine granulosa cells, at all stages of follicular development examined, for each of the ephrin/Eph molecules investigated. Shown in figure 1.3 are representative results obtained following the analysis of a culture of ovulated, murine cumulus granulosa cells grown in vitro for approximately one week, following initial isolation. During the culture period the medium was supplemented with 10% fetal calf serum and no other exogenously added growth factors or supplements. Specifically, an analysis of the EphA1 protein yielded an unusually intense nuclear signal which was evident in every cell examined, fine punctile points of fluorescence were also associated with the cytoplasm/cell membrane in the majority of the cells, figure 1.3A. Signals associated with the EphrinA1protein were more diffusely distributed throughout the entirety of the cell with areas of increased fluorescence intensity associated with the nuclear region, figure 1.3B. Analysis of the EphB1protein showed small, discrete points of fluorescence distributed evenly throughout the cell as well as regions of perinuclear and nuclear staining, figure 1.3C. Examination of the ephrinB1 protein demonstrated a diffuse pattern of fluorescence throughout the cell as well as perinuclear regions of increased staining intensity and, in some cases, fairly bright signal associated with discrete, internal, cellular substructures (endoplasmic reticulum?). Results from the analysis of the EphA2 protein, shown in figure 1.3E, showed an even distribution of fluorescence intensity throughout the cell with slightly increased signal intensity associated with nuclear region of each cell. Antibody control results for this analysis are shown in figure 1.3F. In this case, cells were treated initially with a concentration of nonspecific, primary antibody and secondarily with a solution of FITC-conjugated secondary antibody identical to that used for the analysis of each of the ephrin/Eph proteins. Only low level, nonspecific, background signals were associated with these analyses.

A more detailed and in depth analysis of these and other ephrin/Eph proteins associated with cells of the follicular granulosa cells derived from the human, rat and mouse will be presented elsewhere. Included in these reports

will be the effect(s) of ligand exposure on the staining profiles presented here and *in situ* analysis of the distribution of various ephrin/Eph proteins within intact follicles and ovarian tissues.

THE NATURE OF THE CELLS OF THE FOLLICULAR GRANULOSA

In this chapter an examination of the some of the interleukin molecules, angiogenic growth factors, matrix components and ephrin ligands/receptors synthesized by the cells of the follicular granulosa was undertaken. In virtually each instance, the granulosa not only produced and secreted the individual bioactive molecules discussed, in addition, they also expressed the respective cellular receptors which would enable them to respond effectively to each of the molecules addressed. This is not to meant to imply that the sole purpose for the synthesis and secretion of these factors by the granulosa cells is to effect their own behaviors and functions, that is unlikely to be the case. Rather, the objective of the exercise was to draw attention to and highlight the possibility that autocrine effects for each of the secretory products considered here, synthesized by the cells of the follicular granulosa, is a very real possibility that should no longer be discounted or overlooked.

Without exception, each of the bioactive molecules discussed in this chapter are also effector molecules of endothelial cell function. The parallels between endothelial cells and the cells of the follicular granulosa go well beyond the current presentation. Additional examples of the similarities between endothelial cells and ovarian granulosa cells have been presented previously and include expression of the von Willebrand factor (vWF), CD31 and cKit proteins, rapid internalization of acetylated low density lipoprotein (AcLDL), the ability of human and murine granulosa cells to engage in the formation of tube- or capillary-like structures *in vitro* (Antczak and Van Blerkom, 2000), as well as the expression of receptors and factors that are either directly or indirectly effected by hypoxia, the expression of receptors for estrogen and LH/hCG, and the expression of enzymes utilized in the biosynthesis or inactivation of steroid hormones (Antczak, 2001).

Why do so many parallels between granulosa cells and endothelial cells exist? Are these parallels and similarities clear evidence that the character of the cells of the follicular granulosa is in fact endothelial? No, not necessarily, but the similarities are considerable and they are far too potentially important to continue to be ignored.

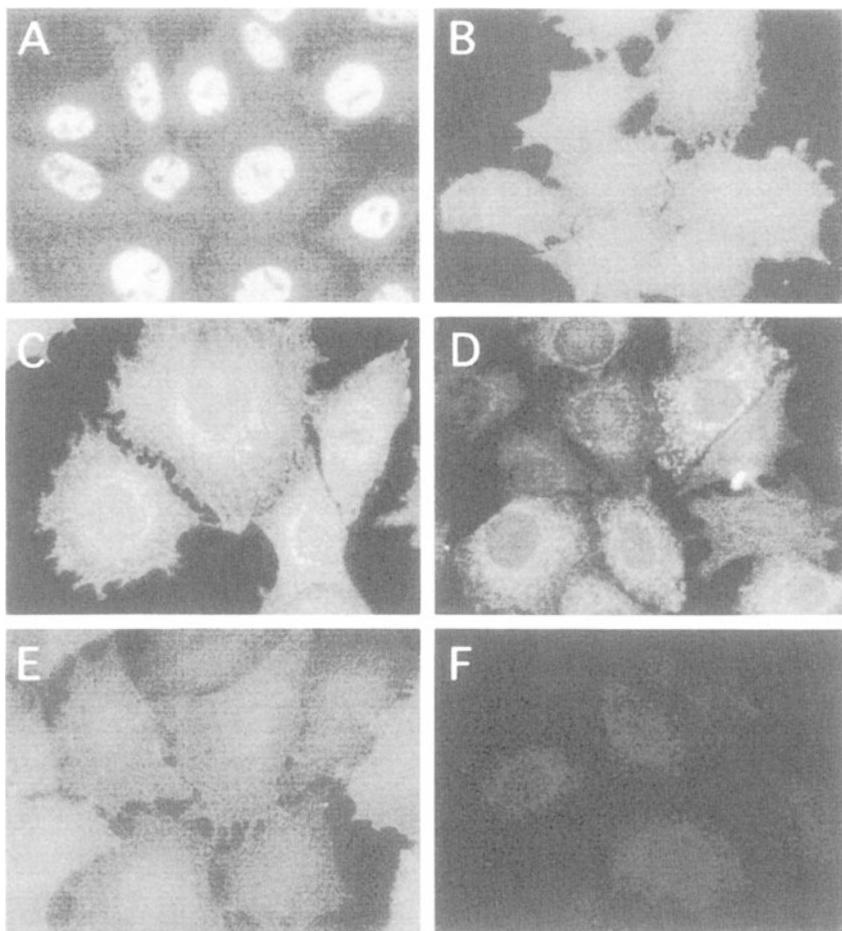


FIGURE 1.3. Results following indirect immunofluorescent analysis of the expression of the EphA1 (A), EphB1 (C), EphA2 (E) and ephrinA1 (B) and ephrinB1 (D) proteins in ovulated murine granulosa cells grown in vitro. Representative antibody control results showing levels of nonspecific antibody interactions and background fluorescence are shown in F. See text for additional details.

Epithelial cell, endothelial cell, does it really make any significant difference? On the surface, the assignment of a specific identity to the cells of the follicular granulosa is of little consequence, if taken no further than a simple designation. However, if pursued to its many potential ramifications, the assignment of a specific cellular identity can be an invaluable tool in furthering the understanding of the biology and function(s) of the granulosa cells, the ovarian follicle, the formation of the corpus luteum and the role(s) of these cells in maintaining the health and integrity of the oocyte. For example,

based on the premise that the cells of follicular granulosa might represent a specialized type of endothelial-like cell, an examination of the Flt-1, Tie-1 and Tek receptor tyrosine kinases was undertaken. Each of these receptor molecules is known to play a fundamental role in endothelial cell biology and function and, as stated previously, each of these receptors is expressed by follicular granulosa cells. If the cells of the follicular granulosa are phenotypically similar to endothelial cells, it would be expected that they would express not only Flt-1 but several other VEGF receptor forms, as is characteristic of endothelial cells. As stated previously, preliminary data suggests follicular granulosa cells also express NP-1 and NP-2 proteins. Once again, NP-1 and NP-2 are receptors not only for VEGF but also for class-III semaphorins molecules. If the cells of the follicular granulosa express NP-1 and NP-2, then by extension, the presence of class-III semaphorins molecules might be expected within the follicle to potentially counterbalance the effects of VEGF₁₆₅ on the follicular granulosa cells. Following this lead, preliminary evidence also suggests that semaphorin molecules are synthesized by the cells of the follicular granulosa (unpublished findings). As a final example, if the cells of the follicular granulosa truly represent a specialized endothelial-like cell population, it might be predicted that they would express members of the ephrin/Eph family of ligands and cellular receptors. The ephrins/Ephs are now known to be signalling molecules of fundamental importance to many endothelial cell behaviors and activities. As shown here, and to be elaborated on in upcoming reports, the granulosa cells express a variety of ephrin/Eph molecules similar to those reported in endothelial cells.

Each of these previous examples underscores the power and utility of a specific cellular assignment/designation for the cells of the follicular granulosa. The assignment establishes a set of expectations which allows for rapid expansion of the knowledge regarding a relatively poorly characterized cell population, the cells of the follicular granulosa, based on the known characteristics of a relatively well characterized, prototypical, cell population, endothelial cells. More important than assigning a specific cellular classification to the cells of the follicular granulosa, is the ability to conceptualize these cells in a way that catalyzes the acquisition of knowledge and understanding of their behaviors and basic biology. That is, after all, the primary objective of the effort.

Finally, if the cells of the follicular granulosa do represent a specialized endothelial-like cell, why endothelial cells? This issue has been addressed extensively elsewhere (Antczak, 2001). In summary, the biology of the ovarian follicle is most unusual in that there is no vascular penetration of the follicle until just prior to or after ovulation, yet during follicular maturation the follicle cells are very metabolically active, they synthesize and secrete a large number of molecules, and they undergo high rates of cell division.

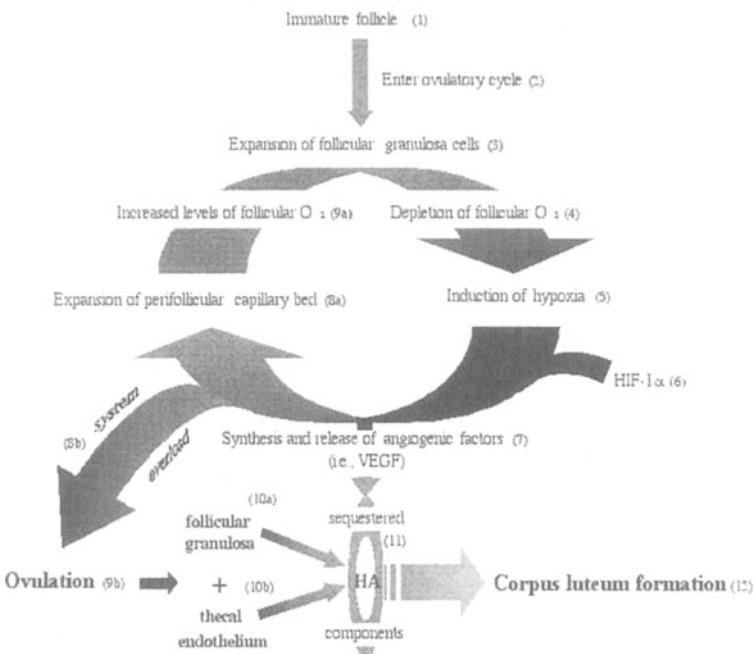


FIGURE 1.4. Possible role of hypoxia during follicular development, ovulation, and corpus luteum formation. An immature follicle (1) enters an ovulatory cycle (2). Hormonal stimulation supports rapid expansion of the follicular granulosa cells (3). The increased number of follicular granulosa cells renders the former level of follicular oxygenation inadequate; follicular O₂ levels decline (4), and hypoxia develops (5). Hypoxia is detected by follicular granulosa cells; hypoxia-inducible factor-1a (HIF-1a) protein accumulates and moves to the nucleus (6). HIF-1a triggers a transcriptional response to hypoxia; angiogenic factors and related components are synthesized and secreted (7). Elevated levels of angiogenic factors support expansion of the perifollicular capillary bed (8a); follicular O₂ levels increase (9a). Increased follicular O₂ supports further expansion of the follicular granulosa; the cycle repeats (3-9a). Alternatively, at a point where supranormal levels of angiogenic factors have been produced, and maximal elaboration of the perifollicular capillary bed has already occurred, the system enters an overload condition (8b) resulting in ovulation (9b). Following ovulation, rapid formation of the developing corpus luteum (12) is assisted through the utilization of stores of angiogenic factors and related components (11), i.e. hyaluronic acid (HA), sequestered during previous intervals (7) and the direct interactions of the remaining follicular granulosa cells (10a) and invading thecal endothelial cells (10b).

Initially, it might seem like this system is poorly designed and prone to the development of, normally unfavorable, hypoxic conditions. However, it is difficult to accept the argument that the follicle is poorly designed – current trends in human population growth alone do not support that contention. Further, the ovary, and the follicles it contains, are entrusted with the fate of

the species, not simply the well-being and health of singular organisms. If anything, the ovary and its follicles would be expected to be honed by evolution to provide an unusually efficient and reliable means of ensuring the passage of successive generations. If there is truly a flaw to be found, it is most likely rooted in our current thinking and lack of understanding of the function of the ovary and its associated follicles.

For example, what if rather than something to be avoided, the development of hypoxia during follicular maturation is an operative element evoked to ensure proper follicular function and specifically calculated to prepare the ovulated follicle for rapid formation of the corpus luteum? A hypothetical model detailing the possible role(s) of hypoxia during follicular development, ovulations and corpus luteum formation is presented in figure 1.4 (this figure represents a modified version of figure 1.2 appearing in Antczak, 2001). If this model is even partially correct, the selection of an endothelial or endothelial-like cell population to reside within the follicle, in the form of the follicular granulosa, would represent an unsurpassed match of functional significance. It is the very nature of endothelial cells that many of their actions and behaviors are driven by relative levels of oxygenation. Endothelial cells are particularly well suited to weather protracted periods of hypoxia by virtue of the many proteins and/or compounds they synthesize which seem to protect them from the phenomenon (see Table 2, in Antczak, 2001). Ultimately, when the level of hypoxia have grown too severe or the duration of exposure too long, it is the nature of endothelial cells to circumvent the difficulty, it is the nature of endothelial cells to correct the hypoxic condition by actively seeking out and providing a source of oxygen to formerly oxygen deprived tissues. It is the charge of endothelial cells to perform these duties irrespective of their location, whether in the tissues bordering a fresh cut or puncture or, potentially, within the bounds of a fully mature ovarian follicle perhaps in association with a process most would term ovulation.

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CHAPTER TWO

OOCYTE-GRANULOSA CELL INTERACTIONS

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INTRODUCTION

The practice of Assisted Reproductive Technology (ART) over the past twenty years has sought to reduce the level of complexity of the human reproductive process into its essential elements, namely, follicular stimulation, antral growth, ovulation induction, fertilization *in vitro*, and embryo replacement into a properly prepared and receptive uterus. Successful outcome, measured by pregnancy rates and live births, has increased progressively and significantly over this period, and in current clinical *in vitro* fertilization (IVF) practice, experienced programs routinely report pregnancy and live birth rates in excess of 40-to-50%. While clinicians are primarily concerned with the production of fertilizable oocytes and embryos that can be transferred or cryopreserved, it is very apparent from the IVF experience that at follicular aspiration, a proportion of oocytes are meiotically immature or if mature, fail to fertilize. In the same respect, a significant proportion of the resulting embryos are developmentally incompetent despite normal morphology and apparently normal performance *in vitro* through the preimplantation stages. Because the primary emphasis of clinical IVF continues to focus on oocyte quantity rather than quality per cycle, the intricacies of molecular and cellular interactions that occur within each follicle between the oocyte and granulosa cell compartments are often not well understood or recognized as critical determinants of developmental competence. Therefore, by reducing "physiological" complexity for clinical purposes, the question of whether and how clinical procedures conform to or modify *in vivo* biology becomes central to understanding the unique potential of each oocyte and embryo generated by IVF. This question becomes of particular relevance in understanding whether current and largely unsuccessful attempts to produce viable human embryos after follicular and oocyte growth from cryopreserved ovarian biopsies, or by maturation *in vitro* of immature oocytes derived from preantral follicles, actually recognize or consider the physiological complexity of the oocyte growth and maturation process.

With respect to the production of competent oocytes, the reduction in 'complexity' noted above for clinical IVF would include the following elements: (1) the consequences of controlled ovarian stimulation (COH) as a pharmacologically driven process causing deviation from a monovular to polyovular process; (2) the legacy of COH cycles with respect to luteal phase deficiencies; (3) the potential developmental effects resulting from bypassing a true ovulatory process linked to increasingly complex signaling events now known to underlie cumulus expansion and oocyte maturation; (4) the need to remove cumulus cells at oocyte retrieval for assessment of oocyte maturation status in cycles where intracytoplasmic sperm injection (ICSI) is used, and (5) supporting perfertilization and preimplantation development through the blastocyst stage in environments lacking relevant somatic cells. This chapter is prompted by the belief that the establishment and maintenance of granulosa cell contacts with the oocyte is vital to both oogenesis and embryogenesis, and that understanding more about these interactions will foster the development of improved techniques for IVF.

Most of the current and standard protocols for human IVF have evolved to maximize the number of follicles available for oocyte retrieval following COH and to assess the stage of oocyte maturation based on the morphology of the cumulus and oocyte. Cumulus removal, typically accomplished by a combination of enzymatic treatments and mechanical stripping, enables direct observation of the first polar body and determination of the stage of meiotic maturation. When related to other measures of follicular maturation including cumulus morphology and endocrine status, a multiparameter assessment of oocyte quality is obtained (Gregory, 1998). Thus, mechanical manipulations of the oocyte assume a fundamental role in the practice of clinical ART. Although it is widely thought that the cumulus and corona cell compartments play no additional role in development beyond ovulation, a precocious disruption of oocyte-somatic cell contacts may compromise oocyte developmental competence through mechanisms that remain unresolved. While removal of cumulus would be expected to facilitate the exchange of media borne substances by diffusion, and perhaps sperm during conventional IVF, the true consequences of dismembering the cumulus-oocyte complex on the outcome of IVF has not been critically evaluated. This issue requires closer inspection as new literature points to novel structural and functional roles for follicle cell-oocyte interactions throughout the course of oogenesis in mammals (Table 2.1; and Albertini et al, 2001).

Recent studies in animal model systems draw attention to an extraordinary level of structural complexity at the interface between somatic follicular cells and the oocyte that varies widely between mammalian species and at different stages of follicular development (Albertini and Rider, 1994; Allworth and Albertini, 1993; Can et al, 1997; DeSmedt and Szollosi, 1991; Familiari et al, 1998; Suzuki et al, 2000; Tesarik and Dvorak, 1982). Moreover, genetic and molecular dissection of a multiplicity of secreted factors, receptors, and

signaling pathways point directly to the existence of sophisticated bi-directional communications systems within the ovarian follicle that directly influence the viability of the follicle and the competence of the ovum alike (Buccione et al, 1990; Canipari, 1994; Deol et al, 2000; Eppig, 2001; Liu et al, 1997; Motta et al, 1984; Ojeda et al 2000). That the regulation of bi-directional communication is dynamic and directly modulated by endogenous gonadotropins or exogenously imposed COH is also becoming apparent (Allworth and Albertini, 1993; Hess et al 1998; Plancha and Albertini, 1984). It is therefore essential to ask how conventional ART protocols satisfy the physiological requirements that underlie the production of viable and developmentally competent oocytes (Trounson and Gardner, 2000; Van Blerkom, 1997).

WHAT IS THE STRUCTURAL BASIS OF OOCYTE GRANULOSA CELL INTERACTIONS?

Established at the very onset of folliculogenesis during the formation of primordial follicles, the structural properties of the interface between granulosa cell and oocyte is modified in form and extent and sustained beyond ovulation in the expanded cumulus mass (Anderson and Albertini, 1976; Hertig and Adams, 1967). The durability, plasticity, and diversity of cell contact relationships throughout the protracted processes of follicular development and ovulation lend testimony to the vital role of germ cell-soma communication and the zona pellucida as a substrate for stabilization of this heterocellular interaction (Aviles et al, 2000; Eppig, 2001; Richards, 2001). Cell junctions of at least two varieties dominate. Gap junctions, composed of connexin subunits that assemble to form channels between the cytoplasm of granulosa and oocytes are a vital structure responsible for transmission of nutrients and signaling molecules (Simon and Goodenough, 1998). Adhesion junctions exploit the cytoskeleton of apposed granulosa cells and oocytes using specialized integral membrane proteins to anchor cells and maintain stable contact zones. These molecular aggregates are now appreciated to mediate signaling via growth factors and receptor tyrosine kinases and as such, adhesive contact sites can be thought of as active signaling domains (Fagotto and Gumbiner, 1996). In order to assemble gap junctions or adhesion junctions at the oocyte surface, specialized cytoplasmic extensions are derived from granulosa cells that breach the zona pellucida and establish contact with the oolemma. Given this disposition, these structures have been referred to as transzonal projections (TZPs) (Anderson and Albertini, 1976; Albertini and Rider, 1994). Their composition, developmental plasticity, and possible functions are only recently becoming clear (Albertini et al, 2001).

TABLE 2.1 DEVELOPMENTAL STAGES AT WHICH OOCYTE-GRANULOSA INTERACTIONS FUNCTION

Stage	TZP Density	Functions
Pre-antral follicle	High	Support oocyte growth/nutrition Transcellular exchange of paracrine factors Establish oocyte polarity Maintain transcriptional/translational activity in oocyte
Pre-ovulatory antral follicle	Moderate	Support acquisition of meiotic competence Support transcriptional repression Mediate maintenance of meiotic arrest Suppress spontaneous luteinization
Ovulatory follicle	High	Facilitate exchange of paracrine factors Mediate corona anchorage for cumulus expansion Remodel zona pellucida matrix Facilitate/enhance sperm hyperactivation /penetration Maintain oocyte polarity/designate site of polar body formation Prevent loss of perivitelline factors Reinforce hyaluronic acid matrix Establish molecular filtration of zona bound factors Regulate deposition and disposition of factors in oocyte

As in the case of neuritic extensions that mediate cell communication in the nervous system, TZPs form an elaborate system of oocyte-granulosa contacts within the ovarian follicle and cumulus-corona complex (Motta et al, 1994; Grosse et al, 2000). TZPs, like neurites, have a core of cytoskeletal elements that includes microtubules, microfilaments, and intermediate filaments (Combelles et al, 2002; Czernobilsky et al, 1985; SeSmedt and Szollosi, 1991; Suzuki et al, 2000). Ultrastructural studies have shown that organelles such as mitochondria occupy TZPs and more recent studies on living follicles show clearly that TZPs are sites of active organelle

translocation for both lysosomes and endosomes (Albertini et al, 2001; Tesarik and Dvorak, 1982) (see Figure 2.1). The abundance of mitochondria located within TZPs further indicates that the generation of ATP within these structures may be important for organelle transport, maintenance of cytoskeleton structural integrity and the provision of energy stores required by the oocyte. Against the background of evidence demonstrating extensive secretion and signaling mediated by paracrine growth factors at the granulosa-oocyte interface, there can be little doubt that TZPs serve a vital role in paracrine communication in the ovary and support the metabolic and biosynthetic needs of the growing and maturing oocyte (Antczak et al, 1997; Antczak and Van Blerkom, 1997; De La Fuente and Eppig, 2001; Smitz and Cortvrindt, 2002). While direct evidence for cumulus-to-oocyte transfer of ATP is lacking, somatic cell secretion and endocytotic uptake by the oocyte or direct transfer through gap junctions represent plausible mechanisms for somatic cell metabolic support. Studies on in vitro matured mouse oocytes have shown that depressed oocyte ATP levels do not influence meiotic progression but do influence embryonic development (Van Blerkom et al, 1995). Whether these later developmental deficits are due to compromised ATP stores of oocyte or somatic cell origin remain to be established.

IS THE OOCYTE-GRANULOSA INTERFACE MODIFIED DURING FOLLICLE GROWTH AND DEVELOPMENT?

With the activation of genes that encode proteins for the zona pellucida during the transition from primordial to primary follicle, the zona becomes a persistent, permanent, and formidable barrier to oocyte-granulosa contact. Assembly of the zona pellucida is recognized to be the by-product of oocyte specific gene expression and secretion of different zona proteins mediated by granulosa cells through as yet ill-defined mechanisms (Eppig, 2001). The ability of granulosa cells to elaborate TZPs and form stable adhesion and gap junction contacts with the oolemma appears to rely on the rapid turnover and remodeling of the zona as folliculogenesis proceeds (Aviles et al, 2000). The density and organization of TZPs changes dramatically during folliculogenesis in the human with a greater prevalence of these structures noted in pre-antral rather than at later stages of follicle development (Motta et al, 1984). Although the factors that regulate formation of TZPs early in follicular development remain unknown, it has recently been suggested that these structures would mediate signaling by various oocyte (GDF9, BMP-15) or granulosa (Kit Ligand) derived ligands known to play essential roles at this stage (Albertini et al, 2001).

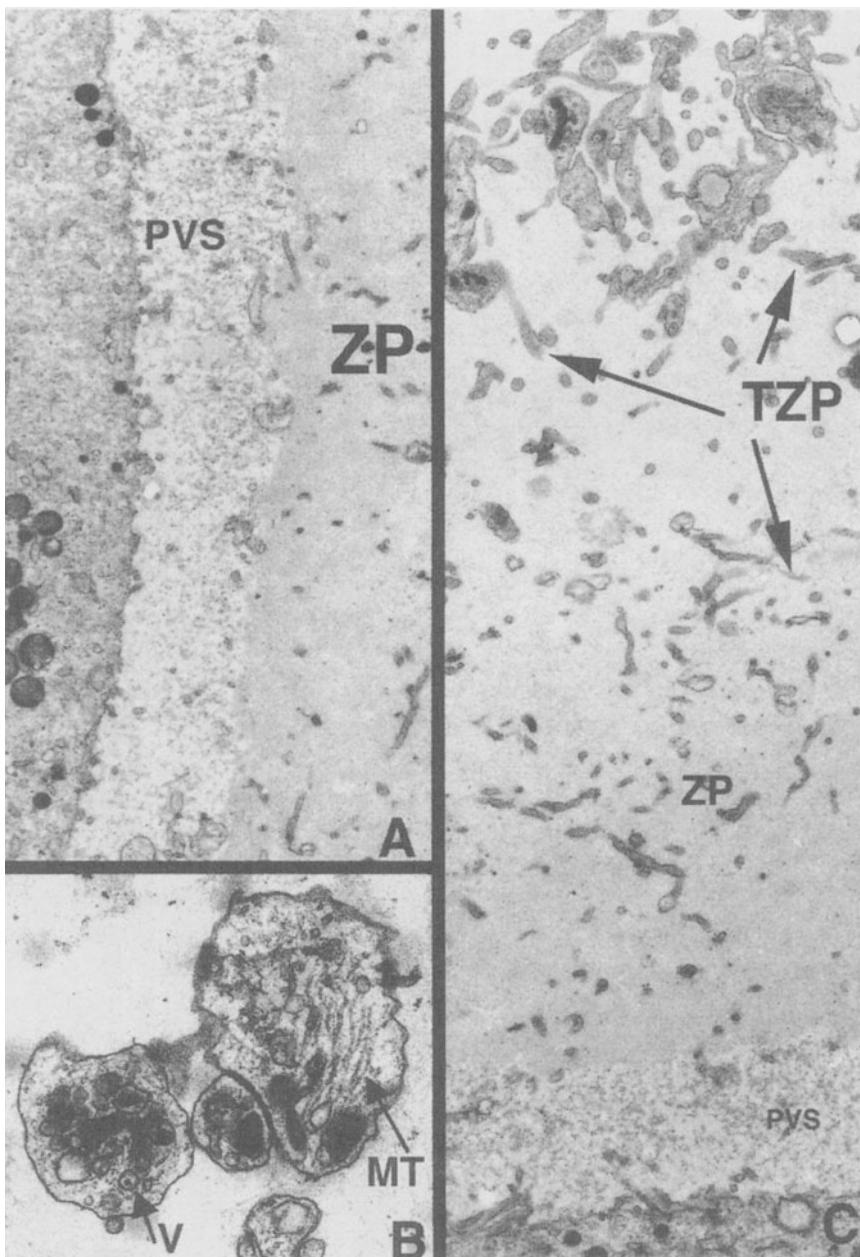


FIGURE 2.1. Electron micrographs of human GV stage (A) and metaphase-2 stage (B, C) oocytes revealing increased density of TZPs following in vitro maturation. B illustrates cross sectional profiles of TZPs containing numerous vesicles (V) and bundles of microtubules (MT). Perivitelline space (PVS); Transzonal projections (TZP).

Since cell adhesion via cadherins also seems to be hormonally regulated in ovarian follicles (Machel et al, 2000), the relative stability of contacts between granulosa cells and the zona or oolemma needs to be more fully determined. In this light, it is interesting to point out that the ability to form gap junctions is often secondary to cells establishing calcium dependent cadherin adhesions, as has been proposed from studies on mammalian ovarian follicles (Albertini and Anderson, 1974; Tesarik and Dvorak, 1982). Since gap junctions that mediate metabolic cooperation essential for oocyte growth largely occur during the preantral stages of follicle development, one would expect that conditions compromising gap junction function early in folliculogenesis would alter oocyte growth and later developmental potential. This idea receives direct support from studies in mice where deletion of the oocyte specific connexin 37 gene resulted in the loss of oocyte-granulosa communication and female sterility (Simon and Goodenough, 1996). Further characterization of this model showed clearly that growth and development of the oocyte were suboptimal, but not lethal, and that follicle development itself was unable to proceed beyond the secondary stage (Carabatsos et al, 2000). Thus, selective disruption of gap junction communication compromises both oocyte and follicle development further reinforcing the mutual dependencies of these processes and their reliance on gap junctional communication.

From a clinical perspective, the relevance of oocyte-granulosa interactions early in folliculogenesis may seem remote to the retrieval of oocytes for IVF. However, COH may directly influence cell communication during cohort recruitment especially at the level of coordinating the protracted development of oocytes following pituitary suppression and exogenous gonadotropin administration. While successful outcomes from IVF cycles are frequent, the possibility that COH influences the normality of oocyte-granulosa interactions deserves further attention, especially with regard to those women who fail to conceive with their own oocytes despite multiple IVF cycles. Moreover, if the transcription and regulation of genes for oocyte granulosa cell junctions are subject to hormonal regulation, it seems likely that perturbations in communication pathways could result from alterations in the follicular hormonal milieu during both preovulatory or periovulatory stages of folliculogenesis. If such alterations have downstream consequences, they could begin to explain the unique and differential competence of embryos within cohorts whose performance in vitro during the preimplantation stages appears equivalent and normal. As noted above, these considerations will also bear directly on ongoing efforts to cryopreserve ovarian biopsies with the intent of generating oocytes/follicles with in vitro follicle technology (Smitz and Cortvriendt, 2002). Thus, central to successfully implementing in vitro strategies to produce developmentally competent oocytes will be the identification of genes involved in mediating oocyte-granulosa interactions and an enhanced understanding of how these gene products are regulated and what functions they have during folliculogenesis. This may be most relevant

in the case of in vitro matured oocytes where clinical success rates are lower than those resulting from oocytes matured in vivo (Trounson and Gardner, 2000).

DO OOCYTE-GRANULOSA INTERACTIONS CHANGE DURING OVULATION AND OOCYTE MATURATION?

The role of oocyte-granulosa interactions during ovulation and oocyte maturation are among the most studied and the most likely to bear directly on the success of human IVF (Hardy et al, 2000; Moor et al, 1998; Trounson and Gardner, 2000). Variations in the degree of cumulus expansion, the retention of corona cells, the size of the perivitelline space, oocyte granularity and morphology of the cumulus or oocyte have traditionally been employed as determinants of oocyte quality used in selection for IVF (Gregory, 1998; Tesarik and Dvorak, 1982). However, the significance of these morphological variations to oocyte maturity status or developmental potential, as assessment criteria at the time of oocyte retrieval remain controversial as they are often arbitrary, subjective and lacking in rigorous explanations as predictive tools (Van Blerkom, 1997). Moreover, since cumulus/corona removal for determination of oocyte maturation status is used in conventional IVF or ICSI, possible detriments associated with somatic cell stripping are unstudied and admittedly intractable from an experimental point of view given the lack of reliable non-invasive measures of oocyte quality. Failure to further explore putative effects of somatic cell removal may have been deemed unnecessary since earlier studies showed that this manipulation did not adversely effect pregnancy outcome (see Trounson and Gardner, 2000 for review). This indicates that corona denudation is not detrimental to many oocytes matured in vivo under standard COH conditions but may heighten concern for the quality and competence of oocytes matured in vitro using abbreviated COH protocols, as noted above.

Thus, we must now rely on data gleaned from recent studies that draw attention to the physiological role of cumulus during the periovulatory period when the reinitiation and completion of meiosis occurs and the cumulus complex undergoes expansion. Cumulus expansion itself is now recognized as a complex series of signaling events involving active transcriptional and metabolic modifications that represent a functional continuation of the oocyte-granulosa dialogue initiated during folliculogenesis (Richards, 2001).

From a metabolic perspective, important changes in levels of ATP, patterns of protein phosphorylation, translation of stored mRNAs, and the generation of reducing equivalents like glutathione (GSH) occur during oocyte maturation in many mammalian species (Moor et al, 1998). These modifications in oocyte metabolism and chemistry are believed to support embryonic cell cycle progression, protect the embryo against oxidative stress, and mediate acquisition of the ability to support pronuclear development upon

egg activation. Moreover, the efficiency with which oocytes undergo enhanced activities of this kind is clearly compromised in the absence of cumulus cells (Liu et al, 1997). Thus, maintenance of cumulus contacts throughout the period of maturation is critical to the support of oocyte metabolism and other biosynthetic activities prior to and immediately following fertilization and perhaps through the preimplantation stages as well. It should be evident then that any procedure that precociously disrupts the metabolic cooperation and communication between oocyte and granulosa will compromise oocyte function even though the consequences of such impairments may not be revealed until later stages of embryonic development, and may not at all reflect on crude measures of oocyte maturity in current clinical use.

What remodeling at the oocyte granulosa occurs during cumulus expansion with respect to cellular connections and signaling? What role does the cumulus extracellular matrix play in the physiological activation of the oocyte or cumulus/corona complex? Again, new findings indicate that oocyte viability and developmental potential rely upon important modifications in cell signaling and secretion of factors that occur during the periovulatory period when cumulus expansion occurs (Antczak and Van Blerkom, 1997; Liu et al, 1997; Moor et al, 1998).

Firstly, the pattern of connections established during oocyte maturation in vitro are modified in a gonadotropin-dependent fashion (Albertini and Allworth, 1993). As shown in Figure 2.1, TZPs increase in density and volume as in vitro maturation of human oocytes proceeds in the presence of recombinant FSH. The question of how these projections form in response to gonadotropins remains unanswered but new insights into the activation of signaling systems is likely to point to direct modification in the stability and/or turnover of cytoskeletal elements. In particular, microtubules occupy TZPs (see Figure 2.2) and form tracks for the movement of organelles such as mitochondria and endosomes. The fact that microtubules form core cytoskeletal elements in TZPs in the human (Figure 2.2A) and that subpopulations of microtubules are post-translationally modified to enhance their stability (Figure 2.2B) is especially interesting (Combelles et al, 2002). To date, no work has been done in human cumulus oocyte complexes (COC) to determine if cytoskeletal modifications induced by gonadotropins are involved in enhancing oocyte quality, as has been shown in other mammals (Allworth and Albertini, 1993), through what appears to be a mechanism facilitating the local secretion and uptake of factors by the oocyte (see below).

Secondly, given the potential dependence on cumulus for adequate ATP generation during oocyte maturation, the influx of mitochondria within TZPs would represent a mechanism for local production and delivery of ATP to the oocyte during the metabolically demanding process of oocyte maturation. In addition, adhesion specializations at the ends of TZPs are probably morphologic manifestations of receptor tyrosine kinase signaling domains that

morphologic manifestations of receptor tyrosine kinase signaling domains that may optimize oocyte quality as a result of receptor activation (Fagotto and Gumbiner, 1996). As discussed below, recent evidence that cumulus cells and oocytes exchange paracrine factors during the periovulatory period would raise the need for the timely passage of substances between the two cell compartments, a role that TZPs have been proposed to provide at earlier stages of folliculogenesis (Albertini et al, 2001).

Whether these connections further serve to establish asymmetries in the distribution of molecules within oocytes remains an intriguing prospect. Recent work has shown clearly that granulosa cell TZPs are sites of STAT3 and leptin production and generate asymmetric patterns of deposition of these and other factors in oocytes and embryos (Antczak and Van Blerkom,

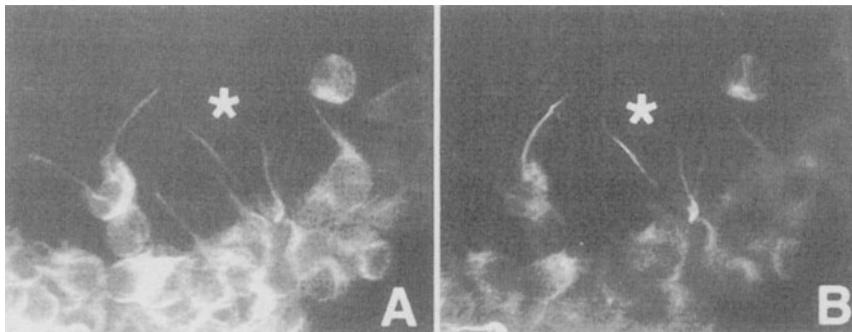


FIGURE 2.2. Confocal micrographs depicting corona/cumulus cell microtubule patterns in a human oocyte at the germinal vesicle stage. Oocyte was retrieved following COH and fixed and processed for the demonstration of total microtubule (A) or acetylated microtubule (B) fractions as described in Combelles et al, 2002. Note presence of TZPs emanating from corona cells and that some, but not all, TZPs contain hyperstable acetylated microtubules (B). * depicts position of oocyte.

1997). The somatic cell source of these materials, coupled with their asymmetric disposition in the oocyte and embryo raises the intriguing prospect that, as in lower animal systems, somatic cell interactions establish asymmetries in the distribution of molecules within the oocyte that underlie axis formation and polarities essential for normal development in the embryo (Edwards and Beard, 1997).

Finally, it has become increasingly clear that complex patterns of signaling in both oocyte and granulosa cells mediate the processes of ovulation, cumulus expansion and maturation of the oocyte. As reviewed by Richards (2001), prostaglandins and steroids elicit changes in cumulus metabolism that are requisite for ovulation itself and have been further proposed to counteract oxidative and inflammatory reactions during, and perhaps beyond, ovulation.

TABLE 2.2 METHODS USED AND POSSIBLE CONSEQUENCES FROM REMOVAL OF CUMULUS AFTER OOCYTE RETRIEVAL

CONVENTIONAL	TARGET	CONSEQUENCES
Hyaluronidase	Cumulus matrix	Enzymatic dissociation of matrix releases cell-cell, cell-zona attachments; increases zona porosity and enhances escape of perivitelline factors; detaches oolemma from zona
Mechanical stripping	Corona cell/zona attachment (integrin/cytoskeleton)	Shear stress ruptures plasma membrane, organelle leakage causes proteolysis, retraction of TZPs increases zona porosity, enhances loss and/or degradation of perivitelline factors; may lead to proteolysis of oocyte surface
ALTERNATIVES		
Divalent cation chelation (EGTA, EDTA)	gap junctions	Rapid uncoupling eliminates gap junction communication and loss of cumulus metabolic support
Hypertonic shock (sucrose)	adhesion junctions	Cell dissociation inactivates signaling at contact sites
Adhesion agonists (RGD, cadherin fragments)	adhesion junctions	Cell dissociation inactivates signaling at contact sites

Whether these changes in metabolic activation of the cumulus also involve deposition of growth factors used during later stages of development are not known but given the fact that disruptive techniques currently in practice eliminate cumulus cells and destroy important matrix-dependent architecture, it is likely that serious perturbations in physiology could result. As summarized in Table 2.2, these would include loss by diffusion of zona or oolemma-bound factors resulting from increased zona porosity. This possibility is particularly relevant to the normality of oocyte function because

it is now known that the normal process of cumulus expansion is attended by remodeling of the zona matrix following TZP withdrawal (Suzuki et al, 2000). Clearly, the challenge ahead is to better define the role of paracrine factors during maturation especially with respect to the stabilizing effects of zona on oocyte receptor-mediated signaling.

WHAT GAPS EXIST IN OUR UNDERSTANDING OF GRANULOSA-OOCYTE INTERACTIONS?

While the above discussion uncovers fundamental features of the structural basis for granulosa-oocyte communication, much remains to be learned regarding how this bi-directional interplay oversees and determines the acquisition of developmental competence in oocytes. The major gaps that remain include the following:

- (1) identification of the role of gap junctions in the maintenance of meiotic arrest, periovulatory resumption of meiosis and meiotic progression to metaphase-2
- (2) clarification of the mechanisms utilized within the cumulus that results in efficient transport of somatic cell factors to the oocyte and their retention, in a polarized fashion, at the oocyte surface or cortical domains (Antczak and Van Blerkom, 1997).
- (3) the need to develop protocols that maintain a homeostatic environment in isolated oocytes that restores and/or propagates the activities of paracrine factors that may alter oocyte/embryo metabolism and enhance developmental potential
- (4) the need to find alternatives for the manipulation of both immature or mature human oocytes so as to optimize conditions for in vitro maturation and embryo production by IVF or ICSI

It should be apparent that until more is learned about the structural and functional basis of granulosa-oocyte communication, gaps in our understanding of the intraovarian support system for oocyte development will continue to hamper progress using current conventional IVF protocols, as well as for procedures being developed for oocyte in vitro maturation, cryopreservation and whole follicle culture (Hardy et al, 2000; Moor et al, 1998; Smitz and Cortvrindt, 2002). It is timely then to consider alternatives to our present approaches in ART that might afford new opportunities for the enhancement of oocyte and embryo quality, bearing in mind the complexity of oocyte-granulosa interactions.

CAN THIS INFORMATION IMPROVE THE PRACTICE OF IVF?

At first glance, it would seem intractable to consider ways of restoring the structural complexity of follicles once enzymatic and physical dissociation of the cumulus are completed. However, a step-wise assessment of these perturbations, as outlined in Table 2.2, gives some direction for future adjustments in protocol. For example, incomplete removal of corona cells following hyaluronidase treatment is not uncommon given the variability observed in the degree of cumulus expansion from COH. Minimizing cell trauma as a result of mechanical stripping would afford retention of a subset of corona cells for sustaining delivery of factors to the oocyte and/or limit their diffusion from the perivitelline space. More conservative dissociation strategies such as these fail to address concerns for ICSI access but may be more appropriate in conventional IVF cases. However, it should be recognized that with current success rates for IVF and ICSI in the 40% or greater range, it is clear that for some proportion of mature oocytes, complete removal of the entire coronal cell complement does not compromise competence. However, it has long been recognized in clinical IVF that the developmental potential of each oocyte is unique, and for some, precocious and complete denudation may have adverse downstream consequences that may or may not be reflected in in-vitro performance during the preimplantation stages. One possibility that may be relevant for investigation is whether blastomere fragmentation, a phenomenon that often effects some proportion of early cleavage stage human embryos within cohorts, may be related to a premature disconnect of signaling between germ and somatic cell compartments.

Other potentially less-damaging means may be implemented to achieve partial or complete cumulus cell removal. Possible alternatives, as shown in Table 2.2. include the following:

- (1) the use of divalent cation chelators. While these agents would be expected to compromise gap junction integrity or elicit undesirable changes in oocyte ionic balance upon prolonged exposure, they may dissociate the more exterior cells of the cumulus with relatively brief treatments (<1 min).
- (2) brief hypertonic shock, typically with 0.1M sucrose, often separates adhesive zones between cells and if used judiciously, may maintain cumulus cell viability and position.
- (3) least explored, but perhaps most promising, would be the treatment with adhesion agonists, small peptides that have been used experimentally to displace selective types of cell adhesions targeted to cell-cell or cell-matrix interactions.

Some combination of these approaches could ultimately lead to less-damaging manipulations of oocytes that retain appropriate cellular relationships required to optimize oocyte quality in clinical IVF and perhaps, rescue from eventual demise, those embryos that develop from oocytes where premature termination of intercellular contact has unexpected downstream consequences.

FUTURE DIRECTIONS

This chapter has focused on the kinds of oocyte-granulosa interactions that serve to coordinate the processes of oogenesis and folliculogenesis. While the complexity of cellular interactions is only beginning to be understood, the likelihood that current practices in the field of human ART may compromise this fundamental determinant of oocyte and embryo developmental competence for some proportion of normal appearing embryos that fail to continue through gestation after uterine transfer. This is likely because at least the following three physiological processes associated with the *in vivo* production of human oocytes are perturbed by virtue of the kinds of manipulations to which this material is subjected: (1) the disruption of TZPs required for bidirectional exchange of factors secreted by oocytes or granulosa cells, (2) the impairment of transport of metabolites and other molecules from granulosa to oocyte via gap junctions, and (3) the interruption of a mechanism used to establish asymmetric gradients of organelles and protein factors that mediate the appropriate spatial allocation of materials within blastomeres. The restoration and maintenance of these organizational principles in human oocytes should, if achieved, substantively enhance outcome in clinical IVF.

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CHAPTER THREE

PERI-FOLLICULAR VASCULARITY: A MARKER OF FOLLICULAR HETEROGENEITY AND OOCYTE COMPETENCE AND A PREDICTOR OF IMPLANTATION IN ASSISTED CONCEPTION CYCLES

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INTRODUCTION

Angiogenesis, the development of new blood vessels, has been described as a foeto-oncogenic event, being a feature of foetal development and associated with the development of tumours. Controlled, cyclical angiogenesis is however, uniquely expressed in the adult female reproductive tract. Angiogenesis in the female tract is expressed in the ovary and endometrium. In the ovary peri-follicular angiogenesis develops with the progression of the follicle to the antral stage, it continues after ovulation with the development of the corpus luteum (CL) and, in the absence of pregnancy, ceases with the regression of the corpus luteum. The cessation of angiogenic events at one site on the ovary, however, is followed by the initiation of angiogenic events at another site in conjunction with the development of new follicles. Angiogenesis in the endometrium follows implantation of the embryo. Here too it is a programd event leading to the development of the placenta but regressing in the event of early pregnancy failure.

Primordial and pre-antral follicles are avascular. Development of the antral follicle from the primordial follicle is accompanied by differentiation of the squamous cells of the primordial follicle to characteristically cuboidal granulosa cells and the differentiation of the thecal cell layers from the ovarian stroma. These cell layers, separated by the basement lamina, are essential to the functioning of the follicle and make a major contribution to the intra-follicular environment, interacting in the hormonal control of follicular development. There are innumerable studies reporting the complex range of activities expressed by granulosa cells which, in addition to their steroidogenic activity, express a range of activities which include the

production of growth factors, cytokines, enzymes and complement regulating proteins (Pellicer et.al., 1998, 2000, Asselin et.al., 2000, Assem et.al., 2000, Moncayo et.al., 1998, Antzak and Van Blerkom, 2000, Taylor and Johnson, 1996): all of which make a contribution to the changing microenvironment of the oocyte - the follicle.

With the progression of the follicle to the antral stage both theca interna and externa acquire a vasculature which comprises a network of capillaries. This network of capillaries, in the follicular phase, is wholly extra follicular. The cyclical changes in ovarian and endometrial vascularity have been recognised for decades and have been the subject of extensive study. Taylor et al (1985) were the first to report a study on ovarian and uterine blood flow characteristics using color Doppler imaging. Numerous studies have followed, often with inconclusive results, in an attempt to identify a correlation between blood flow characteristics of the ovarian and uterine arteries and conception. Thus, Steer et al (1994), Kurjak et al (1991) reported an association between defects in fertility/infertility and sub-optimal perfusion of the uterus in the mid-luteal phase. While other authors e.g. Strohmer et al (1991) reported a lower RI in the uterine arteries on the day of oocyte recovery in women who became pregnant. More recently Engmann and colleagues (1999) have reported a correlation between ovarian and uterine blood flow velocity and outcome from IVF.

PERI-FOLLICULAR BLOOD FLOW

In 1996 Nargund and colleagues reported a correlation between follicular vascularity, measured with color Doppler imaging, and the probability of recovering oocytes. They measured the peak systolic velocity (PSV) and pulsatility index (PI) of peri-follicular blood flow in 94 follicles from a group of 21 women undergoing IVF-ET. They reported a significant correlation between the probability of recovering an oocyte and the detection of peri-follicular blood flow using color Doppler imaging. They also showed a significant increase in the PSV of follicles associated with the subsequent production of an embryo. Thus 62% of oocytes retrieved from follicles with detectable blood flow developed embryos whereas only 48% of oocytes recovered from follicles with no detectable flow developed embryos - suggesting a link between blood flow characteristics and the potential for fertilization. When they assessed embryo quality they found that all embryos from follicles with good blood flow were Grade I or II - according to their definition - whereas 32% of embryos derived from follicles with poor blood flow were grade II or IV.

This study, however, left a number of questions unanswered. The authors did not record the distribution of follicles with different blood flow characteristics among their patient population so that is not possible to determine whether all patients had at least one follicle with detectable flow or

whether there were some women whose follicles exhibited exclusively poor blood flow. Similarly, while 25% of the patient population were seen to have poor ovarian response, with only one or two follicles developed, their results did not record whether this was reflected in blood flow. Finally, there is no indication whether the 4 pregnancies achieved in this group were associated with a particular blood flow pattern.

Color power Doppler imaging (CPDI) is more sensitive than conventional color Doppler at detecting low velocity flow and thus permits measurement of blood flow in small vessels (Rubin et al 1994, MacSweeny et al 1996). It is less dependent upon the angle between the beam and vessels and in areas of multiple vessels the signal is additive so that in a network of small vessels opposing flow directions do not cancel out the signal (Yuval et al 1999.) Thus development of color power Doppler has enabled the measurement of blood flow in capillary systems.

The application of color power Doppler to the measurement of peri-follicular vascularity led, in 1997, to the first report on the correlation between peri-follicular vascularity and pregnancy from IVF (Chui et al.1997) and a reported association between peri-follicular vascularity and dissolved oxygen in follicular fluid (Van Blerkom et al 1997). Both groups reported an increase in aneuploidy relating to poor vascularization of the follicle, with Chui and colleagues reporting a significant increase in triploid embryos where oocytes were derived from poorly vascularized follicles and Van Blerkom and colleagues recording an increase in oocyte aneuploidy, seen as disturbances of the spindle, from poorly oxygenated, poorly vascularized follicles.

FOLLICULAR, OOCYTE AND EMBRYONIC HETEROGENEITY

Despite the worldwide increase in experience and expertise in the treatment of couples with Fertilization in vitro pregnancy rates and implantation rates remain low. The evidence from IVF points to the heterogeneity of follicles, oocytes and embryos and some pregnancy failure undoubtedly relates to the inability of practitioners to identify competent oocytes or embryos from amongst a cohort. Thus, in the UK, implantation rates from IVF-ET remain of the order of 11-13% (HFEA Annual Report, 1999). Further evidence for the heterogeneity of embryos is provided from embryo freezing programs where cumulative pregnancy rates increase following successive embryo transfers. This, in effect, is a means of increasing the number of embryos transferred, albeit over time, and thus circumventing the need for embryo selection.

A competent oocyte is one which has the capacity to develop into a viable embryo. Michael et al.(1993,1995,1996) were the first to report a biochemical marker associated with the activity of follicle cells which gave a measure of oocyte competence. They reported an inverse correlation between conception

from IVF-ET and the expression of the enzyme 11β Hydroxysteroid Dehydrogenase (11β HSD) by granulosa cells in vitro. These observations were remarkable in indicating that the potential for implantation may be determined in the follicle in advance of ovulation. Up until this time it had been assumed that problems of oocyte competence, possibly resulting from an adverse follicular environment, would be evident as a problem of fertilization or early embryo cleavage. The concept that an oocyte could fertilize and develop as a morphologically normal embryo but yet express an underlying deficiency in terms of implantation potential was novel. This observation, that follicular markers could provide a prognostic indicator of implantation potential while being unrelated to fertilization or to embryo "quality", was later confirmed by Gregory et.al. (1994) and Van Blerkom (1996) who showed that the in vitro activity of certain cumulus cells could provide a prognostic indicator of the failure of implantation in IVF.

OOCYTE MATURITY AND COMPETENCE

The increased clinical application of fertilization in vitro techniques to the problems of infertile couples has been accompanied by the use of exogenous gonadotrophins to induce multiple follicular maturation. The success of these techniques, however, depends upon the ability to time egg collections to ensure the retrieval of mature, Metaphase II, oocytes from the ovary in advance of their ovulation.

Most IVF programs use the same, crude, measure of oocyte maturity to determine the time of egg collection i.e. mean follicular diameter measured by vaginal ultrasound accompanied, in some programs, by serum oestradiol measurements. Using these parameters it has been determined that follicular maturation, culminating in ovulation, is usually triggered when follicles reach a mean diameter of 16 - 18mm. In the context of current ART practice, where most programs combine ovarian stimulation with pituitary downregulation with GnRH analogue, oocyte maturation is usually induced in the absence of the endogenous LH surge with exogenous human Chorionic Gonadotrophin (hCG) when several follicles have a mean diameter >18mm.

While there is some debate about the optimum diameter that lead follicles are required to achieve before the administration of exogenous hCG, 16mm, 18mm or >20mm, there is undoubtedly evidence that the mature follicle exerts a paracrine effect within the ovary. Thus mature, Metaphase II, oocytes may be recovered from relatively small follicles, having mean diameters of less than 16mm, provided at least some of the cohort has achieved the optimum size.

However, notwithstanding the success of this method in recovering oocytes which have achieved nuclear maturity there is evidence that the majority of these oocytes are lacking in competence. Thus, despite the recovery of meiotically mature oocytes which have acceptable fertilization

rates the majority of embryos do not have the capacity to implant or to develop to a viable foetus (Human Fertilization and Embryology Authority, Annual Report, 1999) with implantation rates of the order of 11%.

This may relate to the reported high incidence of aneuploidy in human oocytes which may effect more than 25% of morphologically normal oocytes (Van Blerkom, 1994, Delhanty and Handyside, 1995,). It has been suggested that chromosome segregation disorders and spindle defects may result from subtle changes in cytoplasmic physiology such as a reduced intracellular pH (Van Blerkom, 1996). While Gaulden (1992) proposed that follicular hypoxia might have an adverse effect on spindle formation and chromosome segregation.

MARKERS OF IMPLANTATION

It had been expected that the access to human embryos provided by IVF programs would solve the mysteries of conception. However, more than two decades after the first live birth from IVF-ET researchers are still endeavouring to identify the essential components of conception cycles. Embryo morphology and metabolic characteristics have not provided the answer to the search and the need to identify markers of oocyte competence and of embryo viability is self-evident.

The metabolic requirements of the pre-implantation embryo have been extensively investigated (Coates et.al., 1999, Conaghan et.al., 1998, Leese 1998, Orsi and Leese, 2001, Gardner et.al., 1996, 2000, 2001, Erbach et.al., 1994, Biggers 2002 and this volume) in an attempt to identify prognostic indicators of embryo viability. The work of these authors, some of which has been reviewed elsewhere in this publication, has led to a better understanding of the metabolic requirements of the pre-implantation embryo and the production of chemically defined sequential media in an attempt to improve the in vitro conditions. Despite this vastly increased understanding of the embryo's requirements for development and the production of pharmaceutical grade media for routine IVF the heterogeneity of embryos grown in the same media is still expressed in terms of development potential, implantation and pregnancy rates. Therefore the search for refined media does not confer upon a population of embryos a uniform potential for implantation and will not of itself improve the outcome from fertilization in vitro techniques.

The search for markers of implantation has also been directed at the follicle and follicular cells Thus, Gregory et al (1994a, 1994b) demonstrated a positive correlation between the failure of cumulus cells to proliferate in vitro and failure to conceive from IVF-ET with these observations being subsequently confirmed Van Blerkom and colleagues (1996). Yet other research has been directed at aspects of the pre-implantation embryo's development. Scott and colleagues (2000, this volume) and Tesarik and colleagues (2000) reported a correlation between the morphology of the

pronuclear embryo, specifically the alignment of nucleoli within the pronuclei, and the probability of conception from IVF. Whereas others have promoted extended culture of embryos to the blastocyst - a system of "self selection" - to improve pregnancy rates from IVF (Gardner and Lane 1997, Gardner et.al.1998, Jones et.al., 1998, Menezo et al, 1998,2001)

While the identification of embryonic markers would promote better embryo selection and potentially, therefore, improve pregnancy rates the advantages of identifying follicular markers of implantation might be deemed to outweigh those of embryonic markers. Such markers would have the potential to identify non-viable cycles and allow cancellation in advance of egg collection - representing an enormous emotional and financial saving to the couples involved. Such markers might also facilitate 'fine tuning' of stimulation regimes, providing a measure of the physiological status of the follicle and oocyte and refining the timing of egg collection to improve outcome while still providing an objective measure of embryo viability.

PERI-FOLLICULAR VASCULARITY: A MARKER OF IMPLANTATION IN ART?

Chui and colleagues (1997) were the first to report a correlation between peri-follicular blood flow and pregnancy from IVF-ET. They developed a system of quantifying blood flow around the circumference of the follicle at the time of egg collection which depended on the proportion of the follicle circumference which gave a signal on power Doppler angiogram. Using this system they described four grades of follicle with Grade 1 having the poorest flow, <25% of the circumference of the follicle exhibiting blood flow, and Grade 4 follicles having the best blood flow, >75% of the circumference exhibiting blood flow. These initial observations were based on 38 IVF-ET cycles and showed a highly significant association between the failure to conceive and poorly vascularized follicles. Their data showed that, for this series, no pregnancies resulted from cycles where none of the follicles had better than Grades 1 or 2 blood flow i.e. < 50% of the circumference of the follicle showed vascular perfusion. Whereas, they reported a 12.5% pregnancy rate in the group where the best blood flow was grade 3 and a 61.5% pregnancy rate where the best blood flow was grade 4.

Analysis of embryo data from this series supported the observations made by Nargund et.al. (1996b) of the association between follicle vascularisation and the probability of recovering oocytes. Thus, the oocyte recovery rate was 58% from follicles with the poorest ,Grade1, blood flow and 71 - 80% for the remainder of the follicles (Chui et.al.1997). Interestingly the best oocyte recovery was not from Grade 4 follicles, although the differences were not significant for follicle grades 2- 4. Fertilization was also significantly lower for oocytes recovered from Grade 1 follicles, 57%, and significantly more

triploid embryos developed from oocytes which were derived from Grade 1 or 2 follicles: 25% and 31% respectively.

This correlation between poor peri-follicular vascularity and poor outcome from IVF-ET was confirmed in a study of 200 IVF-ET cycles (Bhal et al 1999) and by Borini and colleagues (2001). In Bhal et al's study up to 10 follicles were designated study follicles and their blood flow characteristics (after Chui et.al, 1997) measured at egg collection. Oocytes were processed in individual numbered drops and wherever possible embryos transferred were selected from those which were derived from follicles whose blood flow characteristics had been measured. Data was analysed depending upon the vascularity of follicles, good (blood flow 3 and 4) or poor (blood flow 1 or 2).The results of this study confirmed the heterogeneity of follicles in the same cohort and that peri-follicular blood flow was not related to other parameters of the cycle such as patient age, parity, duration of infertility, duration of stimulation or the dose of FSH to induce stimulation.

When the outcome of treatment, pregnancy, was analysed on the basis of the vascularity of the follicles from which the embryos selected for transfer were derived it showed significantly (34.7%) higher pregnancy rates from embryo transfer where all the embryos were derived well vascularized follicles compared with cycles where embryos were derived from follicles with mixed vascularity (23.5%) or from poorly vascularized follicles (7.3%). These observations were further confirmed by Van Blerkom (1997).

Bhal et.al. (1999) further looked at peri-follicular blood flow and conception in vivo in a series of 261 cycles of stimulated intrauterine insemination (SIUI). In this study pituitary desensitisation was achieved by short downregulation with GnRH analogue and ovarian stimulation with rFSH or hMG. Exogenous hCG was given to induce follicular maturation when one or two follicles had a mean diameter ≥ 16 mm and the peri-follicular vascularity was measured at the time of insemination, 34 - 37 hours post hCG. The vascularity of 792 follicles was measured (after Chui et.al. 1997) and for the purpose of data analysis the cycles were grouped according to the vascular characteristics of the follicles. Group A where all follicles expressed good blood flow, Group B where the follicles were heterogeneous with mixed good and poor blood flow, and Group C where all follicles had poor blood flow. The results again showed a significant correlation between peri-follicular blood flow and pregnancy with the highest pregnancy rate (57%) occurring in the group where all follicles had good blood flow, and significantly fewer pregnancies where there was a follicle population with mixed blood flow (32%) or where all follicles had poor blood flow (11%).

This study further demonstrated a significant association between peri-follicular blood flow and multiple pregnancy. Significantly more multiple pregnancies developing where all the follicles exhibited good blood flow at the time of insemination. This association between blood flow and multiple pregnancy is further confirmed by data from the same centre looking at

pregnancies from ICSI and Perifollicular vascularity (Fig. 3.1: Gregory et al unpublished data).

In this series of 209 consecutive ICSI cycles standard protocols of ovarian stimulation and embryo culture techniques applied. In approximately 50% of cycles blood flow characteristics were not recorded. These cycles gave a pregnancy rate of 32% and a multiple pregnancy rate of 15%. However, where information on peri-follicular blood flow was prospectively used to select embryos for transfer it was found that the pregnancy rate was significantly higher in cycles where the embryos derived from follicles with good blood flow (47% v 32%) with multiple pregnancy rates also being significantly higher (42% v 15%). Conversely where embryos selected for transfer were from follicles with poor blood flow the pregnancy rate was significantly lower (26% v 32%) and there were no multiple pregnancies.

This data confirms the potential of the peri-follicular blood flow to provide an objective marker of embryo “quality.”

Peri-follicular vascularity	Cycles completed	Clinical Pregnancy rate	Multiple pregnancy rate
Blood flow 3- 4	51	47%	42%
Blood flow 1 - 2	49	26%	0
Blood flow not recorded	109	32%	15%

FIGURE 3.1. Peri-follicular vascularity and pregnancy from ICSI

WHAT DETERMINES PERI-FOLLICULAR VASCULARITY?

The observation that peri-follicular vascularity is not uniformly expressed by a cohort of follicles inevitably leads to the conclusion that the development of the peri-follicular vasculature is regulated by events in the follicle. It further suggests that measurements of blood flow characteristics of the ovarian vessels are unlikely to provide a prognostic indicator of conception since measurement of the ovarian artery and stromal flow does not allow for the identification of follicular heterogeneity.

Van Blerkom and colleagues in an extensive study in 1997 showed that peri-follicular vascularity correlated with dissolved oxygen concentrations in the follicular fluid (FF) and with oocyte aneuploidy. Using color Doppler they measured the blood flow characteristics of more than 1000 follicles before aspiration and then measured dissolved oxygen in follicular fluid (FF). Where necessary the FF was centrifuged to remove any red blood cells and vigorous controls applied to ensure that this did not bias the measurements. FF samples

were then stored for subsequent measurement of vascular endothelial growth factor (VEGF). Oocytes recovered from follicles where blood flow characteristics had been noted, but which failed to fertilize were processed and examined for evidence of aneuploidy. Similarly early cleavage embryos, at the 2-cell stage, were examined for the presence of multinucleate blastomeres. Dissolved oxygen concentrations in 1079 samples of FF led them to describe three groups of follicle. The first where the dissolved oxygen was in the range 3-5.5%, the second with a range of 1.5 - 2.5% and the third where dissolved oxygen was $\geq 1.5\%$.

Van Blerkom and colleagues did not find any association between dissolved oxygen concentrations and meiotic maturity or fertilization and in this respect this marker of follicular heterogeneity performs in the same way as other follicle markers of oocyte competence (Michael et al., 1992, Gregory et.al., 1994, Bhal et.al. 1999) The authors did, however, report an association between dissolved oxygen in FF and the capacity of embryos to develop to 6-8 cells with a significant association between low concentrations of dissolved oxygen and a reduced capacity to develop to 6-8 cells. They further reported a 9% incidence of embryos with at least one multinucleate blastomere but significantly multinucleate embryos were derived from follicles with less than 3% dissolved oxygen. The statistically significant association between dissolved oxygen concentrations and abnormal oocyte morphology was also associated with impaired cleavage. They measured VEGF in more than 100 samples of FF from each of the three "follicle groups" and detected VEGF in all FF samples in the range 130 - 180 pM. VEGF concentrations were seen to be unrelated to follicle location, size or fluid volume. However, in follicles with dissolved oxygen $\geq 3\%$ VEGF concentrations were consistently $>270\text{pM}$. Whereas, VEGF concentrations were more variable in follicles with lower concentrations of dissolved oxygen and were consistently low in the follicles which produced dysmorphic oocytes. They showed that both granulosa and cumulus were found to express VEGF with concentrations of VEGF in FF showing an apparent correlation with perifollicular blood flow measurements. Of the 230 oocytes examined which had failed to fertilize they reported that 41% (67/165) of these, apparently morphologically normal oocytes, showed chromosomal scattering or an abnormal alignment of chromosomes on the spindle. They further demonstrated that 92% of oocytes exhibiting a chromosomal abnormality were derived from follicles with a dissolved oxygen $<3\%$.

Gregory et.al. (2001) looked at the role of cumulus in VEGF expression. VEGF in aliquots of the culture medium from cumulus cell culture, derived from individual cumulus oocyte complexes, was measured at intervals over a period of up to 14 days. This study demonstrated that cumulus cells, dispersed *in vitro* by the action of spermatozoa, had the capacity to produce VEGF in culture over an extended period.(Fig 3.2) Concentrations of VEGF were found to differ between different culture drops. However, this was almost

certainly partly due to the differences in cell numbers present in each drop since the cell numbers varied from one COC to another. More significant was the way cumulus VEGF production of individual complexes varied with time (Fig. 3.3).

Day of sampling	Concentrations of VEGF
Egg collection + 1 day	250 - 2000 pg/ml
Egg collection + 7 days	108 - 3300 pg/ml
Egg collection + 14 days	150 - 2000 pg/ml

FIGURE 3.2. VEGF concentrations in medium from cumulus culture (Gregory et.al. 2001)

When serial measurements were made of VEGF concentrations in the medium from individual cumulus complexes from the same cohort significant differences were observed in the activity of cells developed under the same hormonal milieu. The data in Figure 3.3 is in respect of cumulus complexes associated with three embryos selected for transfer in a single cycle which resulted in a singleton pregnancy . This shows that one complex, CC2, was associated with an oocyte derived from a follicle with good blood flow (Grade 3) whereas the remaining two were derived from follicles with poor blood flow (Grade 2). VEGF measurements at egg collection + 1 day did not differ

Cumulus complex (CC) Number.	VEGF (pg/ml) Egg collection + 1 day	VEGF (pg/ml) Egg collection + 3 days	VEGF (pg/ml) Egg collection + 10 days	Peri-follicular blood flow (Chui et. al. 1997)	Embryo morphology at transfer
CC 1	300	560	420	Grade 2	4 cell C+
CC 2	300	1520	1329	Grade 3	4 cell B/C
CC 3	352	880	640	Grade 2	4 cell C+

FIGURE 3.3. Serial measurements of VEGF in the medium from individual cumulus complex cultures associated with embryos selected for transfer in an IVF program (Gregory et.al. 2001)

significantly between the three cell systems. However, cells of CC2, derived from the follicle with best blood flow, produced much higher concentrations of VEGF over a 10 day period, under identical culture conditions, than either of the other cell systems. The cells of CC2 having the capacity to maintain

high concentrations of VEGF in vitro for up to 10 days.. Whereas, CC1 and CC3, while showing an initial increase in VEGF production by Day 3, showed a decline in VEGF by day 10. It may be that these differences in cumulus activity over time are significant. Thus the capacity of cumulus cells to produce VEGF is not terminated at ovulation.

The embryos associated with these cell systems had all developed to four cells by the time of transfer on Day 2 and were seen to have very similar morphology. All showed minimal, fragmentation, scored as C/C+ with one, associated with CC2, being seen to have a small difference in the relative size of one blastomere and therefore scored as B. Thus blood flow characteristics and VEGF production were not reflected by cleavage rates or differences in embryo morphology after 2 days culture. These observations confirmed earlier reports that follicular markers are not correlated with fertilization or embryo morphology assessed on Day 2.

Van Blerkom et al (1997) and Huey et.al. (1999), however found a negative correlation between follicular blood flow and embryo cleavage on Day 3. This is an interesting observation and illustrates the importance in parallel studies of comparing "like with like". When appraising a dynamic system such as the follicle or developing embryo observations on the same events made at different times may well give apparently contradictory results. Thus we have observed that evidence for peri-follicular blood flow on power Doppler angiogram is "lost" as the follicle approaches ovulation with the breakdown of the thecal layer (Gregory et.al. unpublished observations). Depending, therefore upon the timing of peri-follicular blood flow measurements in respect of ovulation it might be possible to miss-interpret the evidence for vascularity in a population of follicles. In our experience the best evidence for peri-follicular vascularity was obtained where the Doppler scan was performed no more than 34 hours after hCG. Changes in the thecal layer as ovulation approaches, particularly at the apex of the follicle, appear to result in a disruption to the capillary system and may therefore give non-representative measures of blood flow.

It is essential too that researchers are aware that these progressive changes in the maturing follicle, from the early antral stage to the ovulatory stage, are accompanied by changes in the activity of follicle cells, granulosa and cumulus, and these will be reflected in differences in the composition of FF. Towards the time of ovulation it would appear that significant changes e.g. in peri-follicular vascularity, may occur over a very short time scale. Gregory et.al (2001) have reported follicular heterogeneity both in respect of peri-follicular blood flow characteristics and of the capacity of cumulus cells to produce VEGF over an extended culture period. Furthermore the capacity of the post-ovulatory cumulus cells to produce VEGF in culture correlated with peri-follicular vascularity measured using color power Doppler after Chui et al (1997) but did not relate to any significant differences in embryo quality. One of the most significant observations to emerge from these studies is the

evidence for the heterogeneous expression of blood flow between adjacent follicles in a single ovary. This is clear confirmation that each follicle is a unique biosystem providing each oocyte with a unique microenvironment during its development. This biosystem functions independently of extraneous factors such as the administration of exogenous gonadotrophins.

WHAT CONTROLS ANGIOGENESIS?

It has been demonstrated that neovascularisation of the corpus luteum (CL) is under the control of heparin binding factors in particular VEGF and fibroblast growth factor which are produced by the granulosa derived cells of the CL (Anthony et.al. 1994, Redmer et.al. 1996). Studies have shown that up to 98% of cells in the developing CL are endothelial (Rodger et.al. 1997). Angiogenesis in the corpus luteum (CL) has been the subject of study for decades. Recently Ferrara et al (1998) demonstrated in the rat that inhibition of VEGF bioactivity resulted in suppression of CL angiogenesis and of progesterone release. This resulted in endometrial abnormalities.

VEGF is an endothelial cell specific mitogen which is known to be a mediator of foetal and tumour angiogenesis and VEGF mRNA expression has been shown to be related to the proliferation of blood vessels. With the evidence from Van Blerkom et al. (1997) and Gregory et al (1998, 2001) for the capacity of granulosa and cumulus cells to produce VEGF it seems that VEGF is one candidate, but probably not the only factor, for initiating angiogenesis in the antral follicle and CL (see Antczak, this volume). Leptin, which has been shown to promote angiogenesis in vitro (Bouloumié et.al., 1998; Sierra-Honigmann, 1998) is present in human follicular fluid (Cioffi et.al. 1997; Karlsson et.al., 1997) and it has been suggested that it may have an angiogenic role in the follicle (Van Blerkom, 1998, 2000).

PERI-FOLLICULAR VASCULARITY AND THE DOMINANT FOLLICLE

In mono-ovulatory species folliculogenesis commences with the recruitment of a number of follicles from the resting pool. Superimposed on this, however, is the mechanism which ensures that only one follicle develops to maturity - the dominant follicle effect. This is regulated by FSH levels which are controlled, by the negative feedback effect of increasing serum oestradiol concentrations, at a level which is sufficient only to maintain the growth of single follicle to maturity.

It has been suggested that selection of the dominant follicle is determined by its vascularity (Suzuki et al) and further suggested that a failure of the peri-follicular vasculature contributes to follicular atresia (Greenwald et al). This is not supported by the evidence from ART, however, where oocytes are recovered from follicles with no measurable blood flow (Chui et.al. 1997,

Bhal et.al. 1999, Van Blerkom et.al.1997, Nargund et.al. 1996b). Interestingly, Bhal et.al. (1999) in a study of peri-follicular vascularity and outcome from stimulated intra-uterine insemination (SIUI) described a group of patients, who despite stimulation with gonadotrophins, were seen on vaginal ultrasound to have produced only a single follicle. When the blood flow characteristics of the single follicle was correlated with pregnancy outcomes then they showed an absolute correlation between poor blood flow and the failure to conceive from SIUI (Fig 3.4).

Perifollicular vascularity	Number of cycles	Pregnancy rate
Good - grades 3 + 4	18	33%
Poor - grades ! + 2	17	0

FIGURE 3.4. Perifollicular blood flow and pregnancy in patients treated with SIUI. (Bhal et.al. 1999).

These results confirmed the observation that the follicular environment is a major regulator of the potential for implantation from conception in vivo as well as in vitro. Significantly they cast doubt on the suggestion that the dominant follicle is the one with the best blood flow since, in this study follicles, developed with poor or no apparent perifollicular vasculature. If, in stimulated cycles, single follicles have the capacity to develop to maturity with a very poor vasculature it suggests the same situation may develop in vivo and this may account for the estimated poor spontaneous conception rate -25% per cycle- for couples with no apparent problem.

THE AGING PROCESS

Data from IVF shows a strong correlation between maternal age and the probability of conception. The most recent data published in the UK (HFEA Annual Report, 1999) shows that women aged over 40 years have <5% chance of conception from IVF. It has been suggested that ovarian ageing is accompanied by changes in granulosa function as well as in the quality of oocyte and embryo. Thus Pellicer et al (1994b) measured progesterone (P) and immunoreactive inhibin production in culture by granulosa cells recovered at the time of egg collection In this study they found a reduction in the steroidogenic activity of granulosa cells from women aged >40 years. Pellicer et al (1988) showed a correlation between poor ovarian response and increased pulsatility index (PI) and resistance index (RI) - which would result in poor blood flow. Thus it would seem that blood flow characteristics in poor responders reduces the chances of conception.

There is evidence from a number of studies that angiogenesis is retarded with ageing and is a feature of retarded wound healing. Thus, diminished angiogenesis in folliculogenesis may be a factor in aneuploidy associated with age while Friedman et al (1997) measured VEGF in FF from older women undergoing IVF and found increased VEGF - associated with hypoxia and a poor rate of conception. This appears to contradict the findings of Van Blerkom and colleagues (1997) who found high VEGF concentration associated with high concentrations of dissolved oxygen in FF and a better prognosis for pregnancy. However the apparent contradictions between these studies may relate to patient selection with Friedman's data being derived from a population of older women. This observation again illustrates the importance of comparing like with like when evaluating published data.

Gaulden (1992) suggested that follicular hypoxia adversely effected spindle formation and chromosome segregation in human oocytes. Where follicular hypoxia results from poor follicular vascularization then this may be the mechanism for aneuploidy in oocytes which has been reported in women of all ages. Van Blerkom et al (1997) suggested that the anoxic status of the follicle is responsible for stimulating VEGF production which then promotes perifollicular angiogenesis. This would in turn lead to increase concentrations of oxygen in FF. Thus, the reported association between poor vascularity, low concentrations of oxygen in follicular fluid and increased aneuploidy may explain the increased age related incidence of aneuploidy. The findings of Friedman et al however, where they found elevated concentrations of VEGF in FF would seem to contradict the evidence that intrafollicular VEGF is responsible for stimulating peri-follicular angiogenesis.

However, Asem et.al. (2000), in a study of the basement lamina in preovulatory follicles from chicken ovaries concluded that the basement lamina was a store or source of biologically active molecules, such as growth factors, growth factor binding proteins, cytokines and matrix metalloproteinases. Whether the basement lamina truly functions in storing these substances or whether it functions as a means of transport of substances to and from the follicle - thus giving the impression of storage - it leads to the suggestion that age related, elevated intrafollicular concentrations of VEGF may result from age related changes in the basement lamina resulting in impaired transport of VEGF to the thecal layer. This would result in a failure of angiogenesis in the thecal layer, the intrafollicular environment remaining hypoxic and thus stimulating production of more VEGF.. The evidence for the poor response of older women to FSH stimulation and the elevated endogenous FSH levels often seen in "poor responders", menopausal women and women with premature ovarian failure provides further indirect evidence for a poor transport system into the follicle. - the resistant ovary effect which is characterised by poor follicular blood flow.

WHY MIGHT PERI-FOLLICULAR BLOOD FLOW BE PREDICTIVE OF IMPLANTATION?

It has been suggested that the significance of peri-follicular blood flow relates to the formation of the corpus luteum (CL) and that the development of an extensive peri-follicular vasculature is a pre-requisite for the development of the vasculature of the corpus luteum. (Bhal et.al.1997a).

Recently Antczak and Van Blerkom (2000) provided evidence that granulosa cells in vitro, express properties which are uniquely associated with endothelial cells (see Antczak, this volume). They described tube-forming activities of granulosa cells in vitro and postulated that this activity in vivo might be essential for the development of the vasculature of the CL. They further suggested that capacity to form tubes in conjunction with their steroidogenic activity could result in the secretion of steroid hormones directly into the blood system and thus potentiate the effect on the endometrium.

There are a number of arguments which suggest that the contribution to the vasculature of the CL cannot be the sole significance for the association between peri-follicular vascularity and conception. If the primary role of peri-follicular vascularity is to provide a competent CL which then expresses properties capable of promoting pregnancy then in superovulated cycles the presence of a single such CL should be sufficient to promote conception without the necessity to transfer embryos derived from follicles with good blood flow. However, the evidence for embryonic heterogeneity points to the necessity to preferentially select for transfer those embryos derived from well vascularized follicles (Chui et.al.,1999, Greory,et.al.1998, Bhal,et.al., 1997 Borini,et.al.2001).

Similarly, oocyte donation programs where the recipients of donated oocytes/embryos are prepared for embryo transfer by a simple combination of exogenous oestradiol and progesterone demonstrate that the CL is not necessary for pregnancy to become established. Furthermore, in this centre's oocyte donation program there is evidence that perifollicular vascularity will determine the probability of conception from oocyte donation as well as from autologous transfer (Gregory et.al unpublished data).Similarly, evidence from frozen embryo transfer cycles, where patients are prepared for frozen embryo transfer using pituitary downregulation with GnRH analogue and the endometrium prepared with a combination of exogenous oestradiol and progesterone, demonstrates that a functional CL is not a requirement for implantation and pregnancy.

The observation that cumulus cells in vitro retain the capacity to produce VEGF for at least 14 days may be significant. These cells invest the oocyte at ovulation and in conception in vivo are dispersed in the fallopian tube by the

action of spermatozoa. It has been suggested that these cells may, while not being essential for pregnancy to occur, promote implantation and pregnancy (Gregory 1998) and there is evidence that implantation rates are better in treatments such as intrauterine insemination and GIFT where the COC remains intact. The cyclic progression from angiogenesis in the ovary to angiogenesis in the endometrium, at the implantation site, is an essential for successful implantation. It may be that the dispersed cumulus cells have a role in initiating the angiogenic events in the uterus which accompany implantation. Antczak and colleagues (1997) described a sub-population of these cells which had the capacity to sequester growth factors by a novel mechanism, with the development of balloon like processes tethered to the cells. They proposed that by this means the cells could concentrate growth factors for a timed release when required and that these packages might have the capacity to be released at one site but exert their effect at a different site. Thus cumulus cells derived from well vascularized follicles and dispersed in the fallopian tube at Fertilization might promote implantation by the release of these balloon like processes which initiate early angiogenic changes in the endometrium.

MEASUREMENT OF PERI-FOLLICULAR BLOOD FLOW - CLINICAL APPLICATION

The selection of embryos for transfer in ART programs remains a largely subjective process, evaluating embryo morphology and cleavage rates. The failure to apply objective measures to the selection of embryos for transfer undoubtedly impacts on the probability of conception from these treatments. It has led to the practice of multiple embryo transfer since this increases the probability of transferring a competent embryo. This has been directly responsible for large numbers of multiple pregnancies and often high rates of fetal morbidity and mortality.

The realization that the heterogeneity of the embryo population is also reflected in follicular and oocyte heterogeneity has opened up a new field in the exploration of markers of implantation. The evidence shows that measurement of perifollicular blood flow, using color power Doppler, allows the prospective selection of embryos for transfer and significantly enhances the outcome from assisted conception treatments, IVF and ICSI, by preferentially selecting for transfer the embryos with the best potential for implantation.

While addressing the problems of embryo selection in a heterogeneous population this does not resolve the situation of women with homogeneously poor blood flow/ embryos whose prognosis is very poor. The application of color power Doppler ultrasound to cycle monitoring does however permit a more scientific approach to the management of treatments. It opens up the possibility of evaluating the potential outcome of a cycle in advance of egg

collection and selectively canceling those cycles where the prognosis is poor. Serial power Doppler measurements of perifollicular blood flow have demonstrated that the vascularity of follicles seems to be established by 9 – 11 days of stimulation and does not significantly change after the administration of hCG to induce follicular maturation. (Bhal and Pugh, personal communication). Thus, in cycles where the perifollicular blood flow is seen to be uniformly poor couples could be counseled as to the poor prognosis for their treatment and advised on the cancellation of the cycle before undergoing egg collection. This would represent an enormous saving, both emotional and financial for the couples concerned. Moreover, evidence obtained from this Unit, in monitoring successive cycles for the same woman, shows that perifollicular blood flow may vary in different cycles. Thus, the capacity to monitor successive cycles with color power Doppler would allow the practitioner to select the best cycle to proceed to egg collection on the basis of objective ultrasound evidence.

It is evident that color power Doppler ultrasound provides a measure of the follicular microenvironment, providing indirect evidence for oxygenation of the follicle and oocyte aneuploidy. These observations raise the question "what determines follicular vascularity?" If it is the oocyte which regulates its own environment and an adverse follicular microenvironment is the result of aberrant signals from an abnormal oocyte the then role of power Doppler in a clinical application may remain passive; providing a measure of "good" and "bad" cycles or "good" or "bad" follicles without providing the means to modify treatments and improve outcome.

However, there is published evidence that cycles may be influenced by the administration of exogenous drugs. Some years after it became accepted practice to control HMG stimulated cycles with GnRH analogue rFSH products became available. However it became apparent that the administration of rFSH in conjunction with GnRH analogue resulted in some women being suppressed to the point where it was not possible to measure plasma LH and the outcome from these treatments was very poor. It has, therefore, been recognized LH is essential for normal folliculogenesis. Thus, the status of the oocyte, its competence, may be affected by an adverse follicular environment induced by cycle manipulation/mismanagement. The application of color power Doppler measurements to cycles in progress might, by allowing the fine-tuning of cycles, lead to the development of improved stimulation protocols providing optimal conditions for oocyte development. Further evidence for the impact of exogenous drugs on the follicle and oocyte is provided by Rubinstein and colleagues (1999). They reported on the effect of low dose aspirin in improving ovarian response, uterine and ovarian blood flow, and pregnancy rates in patients undergoing IVF-ET.

The development of clinical IVF, since the birth of the first baby more than 2 decades ago, has been worldwide. It has however been developed largely independently with practitioners often working in isolation. Thus

practices and results show wide variations. It is evident that oocytes from a single cohort will exhibit marked difference in competence. This may be an inherent quality of the oocyte. Thus the follicular microenvironment, monitored by power Doppler, may be a controlled by the oocyte with abnormal oocytes generating abnormal signals that result in deficiencies in the microenvironment. Thus it might be argued that the outcome of cycles is pre-determined and the practitioner is unable to influence events. However, there is evidence that the inappropriate administration of drugs may have an adverse effect on outcome so that the effective management of ovarian stimulation is an essential for a successful outcome.

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CHAPTER FOUR

DO BIOCHEMICAL PREDICTORS OF IVF OUTCOME EXIST?

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INTRODUCTION

Over the past two decades, several molecules (predominantly steroids and proteins) have been explored as potential predictors of IVF outcome. This chapter begins by reviewing the equivocal data for and against “the usual suspects” (ovarian steroids, gonadotrophins, prolactin, *etc.*) before focusing on the cortisol – cortisol binding globulin (CBG) – 11 β -hydroxysteroid dehydrogenase (11 β HSD) system. This example has been selected to illustrate how independent studies can reach different clinical conclusions, possibly due to differences in the methods used to obtain and assay samples. In the latter sections of this chapter, ongoing molecular studies, which appear to resolve the controversies surrounding assessments of cortisol metabolism as biochemical predictors of IVF outcome, are summarised. The chapter closes by providing an answer to the essential question: “Do biochemical predictors of IVF outcome exist?”

WHY SEARCH FOR BIOCHEMICAL PREDICTORS OF IVF OUTCOME?

Before addressing the focal question for this chapter, it is logical to establish if biochemical predictors of IVF outcome would be useful. The answers lie in the fact that since the birth of Louise Brown in 1978, pregnancy rates following IVF-ET have remained at around 25%. This disappointing figure probably reflects two independent factors. Firstly, a proportion of couples undergoing assisted reproduction would appear to have insurmountable medical problems. Since the probability of conception for these couples is zero percent, inclusion of such couples inevitably reduces the average success rate for most assisted reproduction units. (Clinics which rigorously exclude couples prejudged to have a low probability of conception achieve pregnancy rates in excess of 45%). The second factor is that, to

minimise the risks of multiple pregnancies, either the numbers of embryos transferred has to be limited, or there has to be selective foetal reduction *in utero*. In either case, decisions as to which embryos or foetuses to select are usually based on subjective assessments. For example, in most assisted reproduction units, only those embryos derived from oocytes that have been scored as "mature" are transferred, yet criteria for oocyte maturity vary between different centres. In most assisted conception units, use of subjective criteria for selecting oocytes and/or embryos has little bearing on pregnancy rates.

In light of the issues outlined above, there are two reasons why biochemical predictors of IVF outcome have been so ardently sought. Firstly, they might provide an objective assessment of the likelihood of conception for each couple undergoing IVF, if only on a cycle by cycle basis. Provision of quantitative data to indicate that a particular cycle of IVF is almost certain to fail would assist couples in reaching a decision to abandon that cycle of treatment, saving their emotional and financial reserves for a subsequent attempt at some point in the future. Establishing that a given couple has a very low lifetime probability of conceiving through IVF would allow that couple to make appropriate choices, such as considering adoption or obtaining suitable psychosocial counselling. The second merit of a reliable biochemical predictor is that it may provide an objective index of the developmental potential for an oocyte or embryo, thus assisting the practitioner in the choice of the embryo(s) to select for transfer. Ultimately, a robust predictor of embryo fate would allow the replacement of a single embryo with a high probability of implantation and development, simultaneously improving IVF success rates while avoiding the risks of multiple pregnancy.

THE USUAL SUSPECTS

The search for biochemical predictors of IVF outcome began logically with those molecules known to be involved in endocrine and paracrine control of follicular development, oocyte maturation and ovulation: oestradiol, progesterone, LH, FSH, prolactin, growth factors and cytokines. For each of these hormones, equivocal data have been reported to argue both for and against the use of that biochemical marker as a predictor of IVF outcome.

OVARIAN STEROIDS

The contradictory nature of standard hormonal data in relation to IVF outcome is best exemplified by the specific case of oestradiol concentrations measured at various times prior to, during or after hCG administration. On the one hand, several research groups have reported that conception by IVF-ET is favoured by low serum oestradiol concentrations (typically below 80pg/ml [$<0.29\text{nM}$]) on or prior to IVF cycle day 3 (*e.g.* Andersen & Siebe, 1992;

Costello et al., 2001; Licciardi et al., 1995; Mikkelsen et al., 2001; Smotrich et al., 1995) or on the day of hCG administration (Simon et al., 1995). On the other hand, IVF pregnancies have been correlated with higher serum oestradiol concentrations prior to or at the time of hCG administration (e.g. Chenette et al., 1990; Kavic et al., 2001; Khalaf et al., 2000; Kupesic & Kurjak, 2002; Mettler & Tavmmergen, 1989; Phelps et al., 1998). In between these two extremes, several studies have found no significant association between conception and the serum oestradiol concentrations on any given day prior to hCG administration (e.g. Forman et al., 1991; Hall et al., 1999; Phopong et al., 2000; Schalkoff et al., 1993; Sharara & McClamrock, 1999a, 1999b) while Dor et al. (1992) found that low oestradiol concentrations on the day of hCG administration only predicted improved pregnancy rates in poor responders who yielded less than 4 oocytes following controlled ovarian hyperstimulation.

Appreciating that the timing of single measurements of serum oestradiol may make such assessments of ovarian function unreliable, several studies have investigated the pattern of serum oestradiol concentrations over two or more days, either before or after hCG administration. Although initial studies reported that the rate and pattern of increase in oestradiol concentrations over the first week of hyperstimulation could predict up to a 3-fold increase in the probability of conception (Dirnfield et al., 1985; Leyendecker et al., 1990), contemporaneous studies found no association between the serum oestradiol pattern and IVF outcome (Forman et al., 1991; Hassiakos et al., 1990; Hughes et al., 1990). Likewise, while Reljic et al. (2001) reported a lower oestradiol response to hCG in women that conceived by IVF, Meyer et al. (1999) found this response to be of no predictive value.

Clearly, serum oestradiol concentrations are determined by the steroidogenic activity of multiple ovarian follicles, including those that may be atretic and/or contain immature oocytes. Consequently, one might expect concentrations of oestradiol in follicular fluid to be a better biochemical marker of follicular development and the associated oocyte potential than serum oestradiol concentrations. However, data regarding follicular oestrogen concentrations are as controversial as the serum data already reviewed. For example, while Andersen initially observed lower follicular concentrations of oestradiol in those IVF cycles resulting in pregnancy (Andersen, 1990), he subsequently reported that both free and total concentrations of oestradiol in follicular fluid were unrelated to IVF outcome (Andersen, 1993) as had previously been concluded by Franchimont et al. (1989). Furthermore, while Andersen (1993) reported conception by IVF to be associated with an increased follicular oestradiol:testosterone ratio, Kobayashi et al. (1991) found the follicular ratio of oestradiol:androstenedione to be decreased in follicles yielding oocytes that fertilised and cleaved *in vitro*.

Data regarding serum and follicular concentrations of progesterone are equally contentious. In several studies by a number of independent research

teams, elevation of the serum progesterone concentration above 0.9ng/ml ($>2.86\text{nM}$) prior to hCG administration has been associated with failure to conceive in IVF cycles (e.g. Dirnfield et al., 1993; Fanchin et al., 1993, 1997; Harada et al., 1995; Kagawa et al., 1992; Mio et al., 1992; Schoolcraft et al., 1991; Shulman et al., 1996; Silverberg et al., 1991; Younis et al., 2001). Notwithstanding this relatively common finding, as many published studies have found no correlation between the pregnancy outcome of assisted conception procedures and progesterone concentrations prior to or at the time of hCG administration (e.g. Abuzeid & Sasy, 1996; Edelstein et al., 1990; Givens et al., 1994; Gonen et al., 1993; Hofmann et al., 1996; Huang et al., 1996; Moffitt et al., 1997; Penzias et al., 1992; Sims et al., 1994; Ubaldi et al., 1995). Indeed, three studies have reported that, if anything, IVF pregnancies are favoured (rather than precluded) by pre-hCG elevations in progesterone (Legro et al., 1993; Silverberg et al., 1994; Urman et al., 1999).

Harada et al. (1996) have argued that any association between premature elevation of the serum progesterone concentration and IVF failure reflects an adverse effect of progesterone on the potential of embryos to develop beyond the 4 cell stage. However, the lack of correlation between serum progesterone levels in ovum donation cycles and conception in the recipients has been taken by other groups to indicate an adverse effect of progesterone on endometrial receptivity (Fanchin et al., 1996; Schulman et al., 1996). Interestingly, Eldar-Geva et al. (1997) have postulated that high serum progesterone concentrations prior to hCG administration reflect adrenal hyperactivity, since serum progesterone can (at least in some cases) be normalised by administration of a synthetic corticosteroid (dexamethasone) to suppress adrenal function, facilitating subsequent conception.

As regards post-hCG progesterone concentrations in serum, it has been argued that hCG administration must induce (within 24 hours) a significant rise in progesterone output from luteinizing granulosa cells *in vivo* in order for conception to occur subsequent to ET (Gonen et al., 1993; Prien et al., 1994). However, Prien et al. (2000) argue that this is only the case if a relatively low dose of hCG (5,000mIU) is used to induce final follicular maturation, and is not true if the hCG dosage is increased to 10,000mIU.

Within the follicle, higher progesterone concentrations at the time of ovum pick up appear to be a biochemical predictor of oocyte fertilization and subsequent cleavage (Kobayashi et al., 1991; Mendoza et al., 1999). However, somewhat paradoxically, high follicular progesterone concentrations have also been correlated with failure to conceive by IVF-ET (Franhcimont et al., 1989).

GONADOTROPHINS

Although some studies have reported serum FSH concentrations at baseline and on day 3 of an IVF cycle to be lower in those cycles resulting in

pregnancy (*e.g.* Licciardi et al., 1995; Scott et al., 1989), the majority of studies have found no independent association between FSH concentrations and IVF outcome (Creus et al., 2000; Hall et al., 1999; Padilla et al., 1990; Seibel et al., 1995; Sharara & McClamrock, 1999a, 1999b; Sharif et al., 1998; Watt et al., 2000). Similar equivocal data have been obtained for serum LH concentrations. Whereas a recent study by Barroso et al. (2001) reported lower implantation and pregnancy rates in patients with low LH concentrations and high FSH:LH ratios, prior studies had found decreased pregnancy rates to be predicted by pre-hCG elevation of the serum LH concentration (Munabi et al., 1990) and increased urinary LH excretion (Howles et al., 1987). Other published studies have found no correlation between serum LH and any IVF parameters, including number of oocytes retrieved, fertilization rate or pregnancy rate (Kagawa et al., 1992; Loumaye et al., 1997; Noci et al., 2000).

PROLACTIN

In 1989, Gonen & Casper reported an association between IVF pregnancy rates and the elevation of plasma prolactin concentrations following hCG administration (Gonen & Casper, 1989). Two years later, Oda et al. (1991) reported that plasma prolactin concentrations $>10\text{ng/ml}$ prior to hCG administration were associated with improved fertilization and embryo cleavage rates *in vitro*. In contrast, several studies conducted at the same time found no predictive value in the plasma prolactin concentrations measured at various stages of controlled ovarian hyperstimulation (*e.g.* Hofmann et al., 1989; Hummel et al., 1990; Pattinson et al., 1990).

GROWTH FACTORS

Of the various growth factors known to exert local paracrine/autocrine effects in the ovary, members of the transforming growth factor- β (TGF- β) family, including inhibin and activin, have received the most attention. While follicular concentrations of TGF- β 2, insulin-like growth factor (IGF)-1, platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) all appear to be independent of IVF outcome (McWilliam et al., 1995), increased concentrations of TGF- β 1 in follicular fluid have been associated with conception (Fried & Wrambsy, 1998).

Although serum inhibin concentrations prior to hCG administration have been implicated as a biochemical predictor of conception by IVF (Seifer et al., 1997; Urbancsek et al., 1992), the predictive value of serum inhibin measurements has been undermined by several publications (Creus et al., 2000; Hughes et al., 1990; Penarrubia et al., 2000). Recently, Hall et al. (1999) found that elevated inhibin concentrations were only associated with pregnancy in those patients that had relatively low serum FSH concentrations.

Moreover, enhanced synthesis of inhibin *in vitro* by cultured granulosa-lutein cells has been linked to low serum FSH concentrations *in vivo* in the patients from whom the cells were isolated (Seifer et al., 1996). Given the endocrine role for inhibin in suppressing FSH secretion from the anterior pituitary gland (de Kretser, 1990), it is probable that the association between successful IVF outcome and low serum FSH concentrations (reviewed earlier in this chapter) reflects ovarian output of inhibin which may exert a gonadotrophin-independent local role to influence the developmental competence of the oocyte.

In the mid 1990's, researchers recognised that a healthy preovulatory follicle, characterised by several layers of avascular granulosa cell layers, needs to prepare for an intense period of angiogenesis if it is to generate the highly vascularised corpus luteum required to support pregnancy. This caused several research groups to turn their attention to the concentrations of vascular endothelial growth factor (VEGF) in the follicle. While some found no relationship between follicular levels of VEGF and IVF outcome (Benifla et al., 2001; Manau et al., 2000), others reported high follicular levels of VEGF to be associated with low pregnancy rates (e.g. Battaglia et al., 2000; Friedman et al., 1998). In addition, high serum levels of VEGF may predict ovarian hyperstimulation syndrome (Agrawal et al., 1999; Lee et al., 1997) such that this growth factor may predict increased probability of cycle cancellation.

CYTOKINES

To date, IVF outcome appears not to be associated with the follicular concentrations of interleukin (IL)-6, IL-8, IL-10, IL-11 or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Branisteanu et al., 1997; Geva et al., 1997; Hammadeh et al., 2002). However, increased follicular concentrations of the pro-inflammatory cytokine, IL-1 β appear to be associated with successful oocyte fertilization, subsequent embryo cleavage and pregnancy (Karagouni et al., 1998; Mendoza et al., 1999).

THE CORTISOL-CBG-11 β HSD STORY

In view of the equivocal data regarding conventional endocrine and paracrine modulators of ovarian function as predictors of IVF outcome, the remainder of this chapter focuses on the potential significance of a new candidate biochemical predictor: the adrenal steroid hormone, cortisol.

CORTISOL

Cortisol (referred to clinically as "hydrocortisone") is synthesised in the adrenal cortex. The adrenal glands synthesise two classes of steroid hormone,

the mineralocorticoids and the glucocorticoids, with cortisol being an example of the latter. Cortisol is synthesised from progesterone by the sequential addition of three hydroxyl groups at carbon positions 17, 21 and 11 (Figure 4.1). Due to their similar hydrophobic ("water-fearing") structures, both cortisol and progesterone are transported in serum associated with a globular protein, cortisol binding globulin (CBG).

Cortisol, like all steroid hormones, exerts its actions through an intracellular receptor which mediates effects on the transcription and stability of messenger RNA (mRNA) encoded by target genes. The resemblance in the molecular structures of cortisol and progesterone is reflected in the similarity of their receptors (reviewed by Kumar & Thompson, 1999 and by Whitfield et al., 1999). In addition, these two receptors bind to virtually identical DNA sequences to regulate transcription of the target genes (Funder, 1991; Kumar & Thompson, 1999; Whitfield et al., 1999). The overall consequence of these similarities is that cortisol and progesterone are capable of exerting a number of common actions at the cellular and molecular level.

Over the past 20 years, cortisol has been implicated in the local control of ovarian function. During that time, it has emerged that in a diverse range of fish species, cortisol, 11-deoxycortisol and other hydroxylated metabolites of progesterone are capable of stimulating the maturation and developmental potential of oocytes (e.g. Canario et al., 1989; Kime et al., 1992; King et al., 1997; Mugnier et al., 1997; Patino & Thomas, 1990; Petrino et al., 1993; Pinter & Thomas 1999; Schoonen et al., 1988; Scott & Canario, 1990; Trant & Thomas, 1989). However, in porcine oocytes, glucocorticoids appear to inhibit meiotic (but not cytoplasmic) maturation (Chen et al., 2000; Yang et al., 1999).

In women undergoing assisted conception, follicular cortisol concentrations have been reported to increase as follicular rupture approaches (Andersen & Hornnes, 1994; Harlow et al., 1997), although the significance of this phenomenon is unclear at present. Whereas two studies have reported higher cortisol concentrations in follicles containing mature oocytes (Fateh et al., 1989; Jimena et al., 1992), others have found no association between this glucocorticoid and oocyte maturity (Andersen & Hornnes, 1994; Mantzavinos et al., 1997). More importantly, follicular cortisol concentrations do not appear to predict successful oocyte fertilization (Bider et al., 1998; Fateh et al., 1989; Mantzavinos et al., 1997) nor pregnancy (Andersen & Hornnes, 1994).

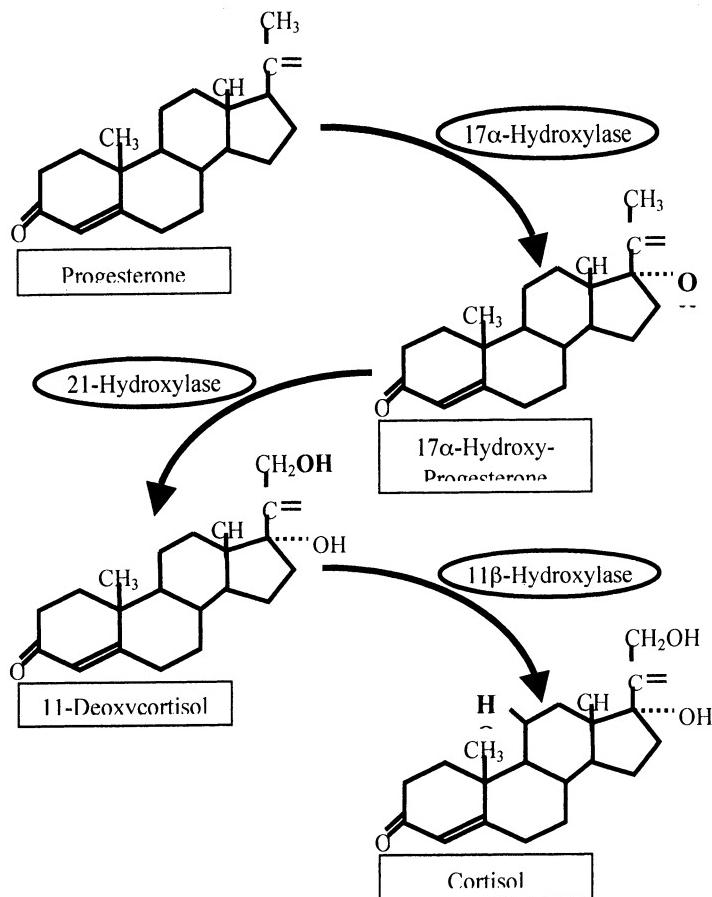


FIGURE 4. 1. Adrenal synthesis of cortisol

BINDING GLOBULINS

In his 1990 study, Andersen measured the serum and intrafollicular concentrations of both CBG and of sex hormone binding globulin (SHBG)(Andersen, 1990). Levels of CBG were significantly higher in follicles aspirated in successful IVF cycles than in those that failed, leading Andersen to conclude that CBG may be a biochemical predictor of IVF success. In the face of equal follicular cortisol concentrations, an increase in the CBG concentration might be expected to decrease the bioavailability of cortisol, compromising the ability of cortisol to enter target cells and interact

with glucocorticoid receptors. However, this situation is complicated by the fact that CBG binds both cortisol and progesterone (Andersen & Hornnes, 1994). Due to the high concentrations of progesterone in follicular fluid, only a small proportion of the cortisol inside the ovarian follicle remains bound to CBG such that a modest increase in the CBG concentration may have little or no effect on the free (bioactive) concentration of cortisol.

THE 11β HSD ENZYMES

As mentioned previously, steroid hormones act by binding to intracellular receptors which can interact with DNA and mRNA to influence expression of proteins in target cells. Although the structure of the steroid-binding domain of each receptor places certain constraints on the types of steroid that can bind to and activate that receptor, this system for receptor specificity is not fool-proof. For example, *in vitro*, the “mineralocorticoid receptor” is unable to discriminate between the structure of the physiological mineralocorticoid, aldosterone (central to the control of water and electrolyte balance) and the glucocorticoid, cortisol (Arriza et al., 1987; Funder et al., 1988). In such cases, an additional mechanism is required to increase the specificity of interaction between the receptor and candidate ligands. This usually takes the form of one or more intracellular enzymes that modify the steroid structure and, in so doing, either increase or decrease its affinity for the binding domain of the receptor.

The significance of this phenomenon is best explained by analogy to a night-club where the cashier (the steroid-binding domain) is rather weak and so relies on the security staff (the steroid-metabolising enzymes) to discern whether people trying to enter the club (the receptor) should be admitted or denied access. In this event, were the security staff to go off duty or be overwhelmed by a sudden rush of undesirable patrons, then the cashier would be unable to cope and mayhem would ensue. Likewise, if the enzymes that protect the steroid receptors are in any way compromised, there is increased binding of steroids (even to inappropriate receptors) and enhanced effects on the expression of target genes.

In the case of the mineralocorticoid and glucocorticoid receptors, the access of cortisol is controlled by isoforms of the enzyme 11β -hydroxysteroid dehydrogenase (11β HSD)(Bush et al., 1968; Monder, 1991; White et al., 1997). These enzymes catalyse the reversible removal of two hydrogen ions, so inter-converting the active steroid cortisol, with its inert metabolite, cortisone (Figure 4.2.).

In the early 1990s, the enzymatic oxidation of cortisol to cortisone was first reported in human granulosa-lutein cells aspirated from the follicles of women undergoing IVF (Michael et al., 1993a; Owen et al., 1992). Shortly after, we reported a potentially predictive association between the rate of 11β HSD-mediated oxidation of cortisol and IVF outcome (Michael et al.,

1993b; 1995). In these studies, 11 β HSD enzyme activities were assessed *in vitro* by quantifying net oxidation of ^3H -cortisol to ^3H -cortisone over 4 hours. While cells from two thirds of patients exhibited moderate to high levels of 11 β HSD enzyme activity, cells from one third of the patients had very low levels of 11 β HSD activity, below the assay detection limit. Whereas none of the patients with detectable ovarian 11 β HSD activities subsequently conceived, low 11 β HSD enzyme activities were associated with a pregnancy rate of 64% (Michael et al., 1993b, 1995).

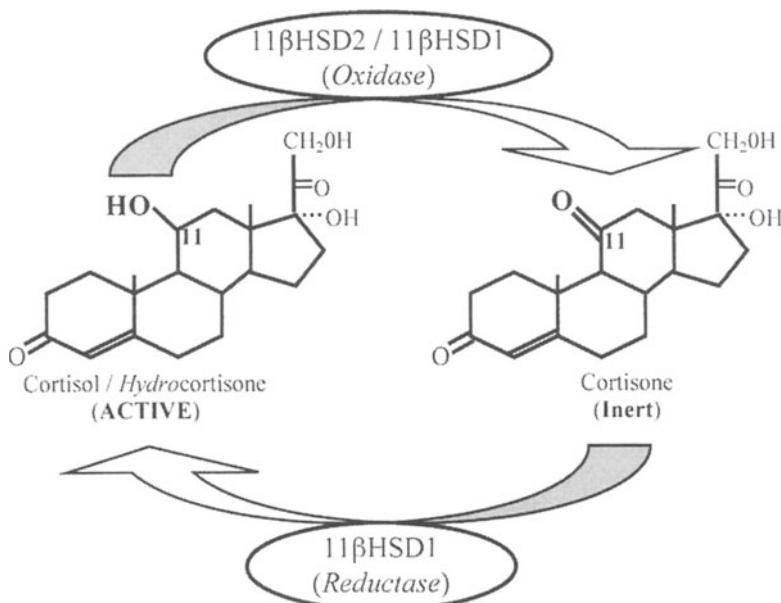


FIGURE 4.2. Cortisol-cortisone inter-conversion by the cloned isoforms of 11 β HSD.

Following the publication of this encouraging data, two independent research groups explored this potentially predictive relationship (O'Shaugnessy et al., 1997; Thomas et al., 1998). Although neither of these groups found a significant association between ovarian 11 β HSD activities and IVF outcome, the study of Thomas et al. did show a trend for mean rates of cortisol metabolism to be lowest in those cycles resulting in pregnancy and highest in cycles with total fertilization failure, with intermediate enzyme activities in non-conception cycles.

On closer inspection, differences in the methods used to assess ovarian 11 β HSD activities may have had a bearing on the relationship between this biochemical marker and IVF outcome. For example, in the study of

O'Shaugnessy et al. (1997), granulosa cells were frozen at the time of follicular aspiration, and enzyme activities were subsequently assayed in thawed cells. Data from a number of laboratories indicate that 11 β HSD enzymes are sensitive to damage by freezing and thawing such that this may have compromised the measurement of ovarian 11 β HSD activities.

A second significant difference is that in our initial studies of ovarian 11 β HSD activities, it had been necessary to store cells in follicular fluid and/or follicular flushing medium at 4°C for up to 3 days prior to isolation. After isolation on Percoll, granulosa cells were then cultured for 3 days in serum-supplemented medium (to facilitate re-establishment of the luteinized phenotype) before assessing 11 β HSD activities on day 4 of culture (Michael et al., 1993a, 1993b, 1995). Neither the study of O'Shaugnessy et al. (1997) nor that of Thomas et al. (1998) stored or routinely cultured granulosa cells prior to assessment of cortisol-cortisone inter-conversion. This raises the question as to whether cell storage and/or culture may be necessary to reveal a relationship between cortisol metabolism and IVF outcome. In support of this postulate, when we have assessed ovarian 11 β HSD activities in freshly isolated human granulosa cells, and without pre-culturing cells to facilitate luteinization, we have found no association between enzyme activities and IVF success or failure. Additionally, we have found that culturing granulosa cells in the presence of serum for 3 days prior to enzyme assay markedly decreases the rate of 11 β HSD-mediated cortisol oxidation; a finding which was first reported by Thomas et al. (1998). Hence, for reasons explained towards the end of this chapter, we propose that the decline in ovarian 11 β HSD activities with granulosa cell storage and/or culture appear to be necessary to reveal a relationship between *in vitro* measurements of 11 β HSD activities and IVF outcome.

In the late 1990's, following the cloning of two distinct isoforms of both the rodent and human 11 β HSD enzymes (Agarwal et al., 1989; Albiston et al., 1994; Rajan et al., 1995; Tannin et al., 1991; Zhou et al., 1995), a number of molecular studies contributed to the debate regarding the potentially predictive role for 11 β HSD in human IVF. Research conducted at the MRC Human Reproductive Sciences Unit in Edinburgh established that in human granulosa cells, the dominant enzyme isoform alters as cells luteinize following exposure to hCG (Tetsuka et al., 1997; Yong et al., 2000). Prior to exposure to LH/hCG, granulosa cells express mRNA encoding a high affinity isoform of 11 β HSD (11 β HSD2) which appears to act exclusively as an oxidase enzyme using nicotinamide adenine dinucleotide (NAD⁺) as an enzyme cofactor. However, following exposure to hCG, luteinizing human granulosa cells no longer express 11 β HSD2, but instead express the gene encoding the relatively low affinity isoform of 11 β HSD (11 β HSD1). This isoform appears to act predominantly as a reductase enzyme with a preference for nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor

(Tetsuka et al., 1997; Yong et al., 2000). Data from other laboratories support expression of 11 β HSD1 in luteinized human granulosa cells, but also indicate the existence of a novel, biochemically distinct isoform of 11 β HSD in human granulosa-lutein cells (Michael et al., 1997; Smith et al., 1997).

The molecular switch in 11 β HSD enzyme isoforms as granulosa cells luteinize in pre-ovulatory follicles may explain the observations of a potentially predictive relationship between the oxidative activity of 11 β HSD and IVF outcome. Since initial assays of 11 β HSD activity measured the *net* oxidation of cortisol to cortisone, levels of cortisol metabolism would have been expected to be highest in granulosa cells from immature follicles, expressing the oxidative 11 β HSD2 isoform with only limited expression of the 11 β HSD1 reductase enzyme. In contrast, in mature follicles that had responded to hCG administration, increased expression of the 11 β HSD1 enzyme would be anticipated, with concordant suppression of 11 β HSD2 expression. The simultaneous increase in the reduction of cortisone, accompanied by a decrease in the oxidation of cortisol, reflected as an overall decrease in the *net* oxidation of cortisol to cortisone, may thus have provided a biochemical marker of follicular maturity.

In 1996, researchers at the Western General Hospital, Edinburgh, reported production of transgenic “knockout” mice lacking 11 β HSD1 (Kotelevstev et al., 1996). Despite the absence of this enzyme, these mice showed normal fertility (Holmes et al., 2001; Kotelevstev et al., 1996), causing some researchers to question the reproductive significance of 11 β HSD in the ovary. However, there are several reasons why the findings with the 11 β HSD1 knockout mice have to be interpreted with caution. For example, one of the intrinsic problems of targeted gene deletions is that one or more related proteins might adopt the role of the deleted gene product and so compensate for the chronic absence of the deleted protein. In this specific instance, 11 β HSD2 and/or as yet uncloned isoforms of 11 β HSD may have assumed the biochemical function of the deleted 11 β HSD1 protein. Clearly mice with co-deletion of both cloned isoforms of 11 β HSD would address this issue, but attempts to date to produce such animals have been unsuccessful, indicating that the double knockout may be embryonic lethal. A second problem lies in the assumption that chronic genomic deletion of the 11 β HSD1 protein in the ovary would model the natural circumstances under which ovarian 11 β HSD activities are low. It may be that low enzyme activities as measured in human granulosa-lutein cells reflect the presence of local enzyme inhibitors, which influence IVF outcome independent of the suppression of 11 β HSD-mediated glucocorticoid metabolism (discussed in the penultimate section of this chapter). As more data accrue, it seems increasingly likely that in the initial studies, 11 β HSD-mediated oxidation of cortisol to cortisone was a surrogate marker of other ovarian events that would not be reproduced by transgenic knockout of a single isoform of 11 β HSD. Finally, given the major differences

in reproductive biology between rodents and primates, excluding a role for 11 β HSD1 in the fertility of mice does not necessarily exclude a role in humans.

Returning to human IVF, subsequent studies by Smith et al., (1997, 2000) at the University of Bristol found the levels of expression of mRNA encoding 11 β HSD1 in human granulosa cells to correlate with the probability of conception in women undergoing IVF (Smith et al., 1997). However, levels of mRNA do not necessarily equate to levels of the encoded enzyme protein and to enzyme activities. The data summarised in the following sections of this chapter all support the hypothesis that the potentially predictive relationship of ovarian cortisol-cortisone inter-conversion and IVF outcome, only observed under defined conditions, reflects ovarian events that control the activity of the 11 β HSD enzyme(s), probably at the post-translational level.

Within the Leydig cells of the testis, 11 β HSD1 appears to serve a crucial role in preventing the inhibition of testosterone biosynthesis by glucocorticoids (Gao et al., 1996; Ge & Hardy, 2000; Monder et al., 1994; Phillips et al., 1989; Sankar et al., 2000). As mentioned previously in this chapter, testicular cells may also express a novel, high affinity isoform of 11 β HSD (Ge et al., 1997), akin to the biochemical activity observed in human granulosa-lutein cells (Michael et al., 1997). In 1997, 11 β HSD-mediated metabolism of cortisol was demonstrated in human spermatozoa and other cellular components of human semen (Nacharaju et al., 1997). Moreover, the net oxidative activities of 11 β HSD in human semen samples with low sperm counts and/or a high percentage of morphologically abnormal sperm were found to be 10-fold higher than those in semen samples with high sperm counts and/or good sperm morphology. It appears, therefore, that low net conversion of cortisol to cortisone in gonadal cells, whether germ cells or somatic cells, may indicate better prospects for IVF outcome in both sexes.

FOLLICULAR CORTISOL:CORTISONE RATIOS

Following the studies of ovarian 11 β HSD activities *in vitro*, we attempted to assess enzyme activities *in vivo* by measuring concentrations of cortisol and cortisone in follicular fluid. *In vivo*, ovarian 11 β HSD activities should be reflected in the follicular ratio of cortisol:cortisone. Assuming cortisol to be the predominant enzyme substrate and cortisone to be the major enzyme product, high follicular cortisol:cortisone ratios would indicate low ovarian 11 β HSD activities, whereas low cortisol:cortisone ratios should reflect high rates of cortisol oxidation by ovarian 11 β HSD (Andersen et al., 1999; Michael et al., 1999).

In 1999, we reported a preliminary study in which follicular cortisol:cortisone ratios were significantly higher in follicular aspirates from successful IVF-ET cycles than in those follicular fluid samples obtained from

non-conception cycles (mean ratios = 9.7 versus 6.9, respectively). As regards predictive value for IVF outcome, follicular cortisol:cortisone ratios greater than 7.7 (the mean ratio irrespective of IVF outcome) were associated with a 50% pregnancy rate, whereas ovarian cortisol:cortisone ratios less than 7.7 were associated with a pregnancy rate of only 7.6% (Michael et al., 1999). Based on the assumptions defined above, such data support a potentially predictive, inverse correlation between 11β HSD-mediated cortisol metabolism and the probability of conception by IVF.

To date, all of the studies conducted in my laboratory have used samples obtained from gonadotrophin-stimulated IVF cycles. Since LH and hCG have both been shown to increase the expression of 11β HSD1 (Tetsuka et al., 1997, 1999), the ratio of cortisol:cortisone may have been influenced by the protocol for controlled ovarian hyperstimulation. However, strikingly similar data have very recently been reported from a study of follicular cortisol:cortisone ratios in women undergoing natural cycle IVF; ratios were highest in those IVF cycles resulting in pregnancy and lowest in those where all oocytes failed to fertilize *in vitro*. Furthermore, follicles with cortisol:cortisone ratios greater than the outcome independent median value of 5.9 were associated with a pregnancy rate of 58% versus only 13% for those follicles with lower cortisol:cortisone ratios (Keay et al., 2002). The ability of follicular cortisol:cortisone ratios to predict conception does not, therefore, appear to be effected by the use of gonadotrophins to induce multiple follicular development.

In contrast to the studies reviewed above, Andersen et al. (1999) found no significant relationship between follicular cortisol:cortisone ratios and IVF outcome. This finding raises the possibility that the relationship between an *in vivo* index of 11β HSD activity and IVF outcome may be influenced by subtle differences in clinical treatment and/or experimental protocols, as appears to be the case for direct measurements of ovarian 11β HSD activities *in vitro*.

In view of the questions raised concerning the predictive value of follicular cortisol:cortisone ratios and IVF outcome, we have just completed a study of this relationship in a series of 80 IVF patients. In this study, the upper quartile value for the follicular cortisol:cortisone ratio for all samples, irrespective of IVF outcome, was 11.2. Strikingly, 18 of those 20 follicular samples with cortisol:cortisone ratios greater than 11.2 were associated with pregnancy, whereas *all* of the 60 follicular fluid samples with cortisol:cortisone ratios less than 11.2 were obtained from unsuccessful IVF cycles (Thurston et al., 2002a). These data add significant support to the hypothesis that ovarian cortisol-cortisone inter-conversion, mediated in the follicle by one or more isoforms of 11β HSD, can predict IVF outcome, if only under defined circumstances.

OVARIAN ENZYME MODULATORS

With renewed belief that ovarian 11β HSD activities *can* predict the probability of conception by IVF-ET, we have turned our attention as to why the direct measurements of ovarian 11β HSD activities *in vitro* might be influenced by:

- culture of human granulosa cells in serum-supplemented medium for 3 days prior to enzyme assay
- storage of aspirated cells for up to 3 days at 4°C in follicular fluid and/or follicular flushing medium prior to the isolation and culture of cells.

In 1996, we reported evidence that human granulosa-lutein cells synthesise and secrete paracrine inhibitors of cortisol metabolism (Michael et al., 1996). A candidate for the local inhibition of ovarian 11β HSD activity would be progesterone which is the major secreted product of luteinized granulosa cells and which is an established inhibitor of the cloned isoforms of 11β HSD, particularly 11β HSD2 (*e.g.* Souness & Morris, 1996; Souness et al., 1995). Since synthesis of this steroid increases progressively as granulosa cells luteinize over 3 days in serum-supplemented culture (*e.g.* Fowkes et al., 2001), increasing production of progesterone could account for the decline in the rate of cortisol metabolism seen in many granulosa cell samples over the first 3 days of culture. Recently, we tested this hypothesis by suppressing basal progesterone output from cultured human granulosa cells using the cytochrome P450 enzyme inhibitor, aminoglutethimide. On the one hand, blocking progesterone synthesis elevates ovarian 11β HSD activities confirming that progesterone acts as a local inhibitor of cortisol-cortisone inter-conversion in cultured human granulosa-lutein cells. On the other hand, even granulosa cells incubated continuously with aminoglutethimide exhibit a progressive decline in the levels of 11β HSD activity despite increasing expression of 11β HSD1 protein, indicating that such cells must be producing an enzyme inhibitor other than progesterone.

Given that human granulosa-lutein cells appear to produce paracrine inhibitors of 11β HSD activity during culture, we are currently investigating whether these enzyme modulators are present in the follicular fluid in which our granulosa-lutein cells are stored for up to 3 days prior to isolation. In order to enrich any enzyme inhibitors present in follicular fluid, we have adopted a method used by Morris and colleagues to resolve endogenous inhibitors of 11β HSD from urine (Lo et al., 1997; Morris et al., 1992). Samples of follicular fluid are loaded onto C18 Sep-pak chromatography cartridges, from which compounds of increasing hydrophobicity are sequentially eluted using a gradient of increasing methanol concentrations. Each fraction is then tested for its ability to modulate either NADP⁺ or

NAD⁺-dependent oxidation of cortisol using rat kidney homogenates as a source of both cloned isoforms of 11 β HSD.

Using this approach, we have confirmed that human follicular fluid samples contain hydrophobic molecules, eluted at methanol concentrations ranging between 55 and 90% (vol/vol), which are capable of significantly inhibiting NADP⁺-dependent 11 β HSD activity (but not NAD⁺-dependent cortisol oxidation) within 1 hour. Those compounds eluted by 80% methanol achieve the maximum enzyme inhibition, suppressing NADP⁺-dependent 11 β HSD activity by up to 88% relative to the control enzyme activity (measured in the presence of the appropriate concentration of methanol alone). Surprisingly, this technique has also revealed that follicular fluid contains hydrophilic compounds, eluted at 0% and 10% methanol, which can stimulate both NADP⁺-dependent and NAD⁺-dependent 11 β HSD activities by as much as 2.7-fold within 1 hour (Norgate et al., 2002).

Having found that human follicular fluid contains endogenous stimuli and inhibitors of 11 β HSD activity, ongoing research is addressing whether the levels of these compounds determine the follicular cortisol:cortisone ratios and/or serve as a novel biochemical predictor of IVF outcome. The ratio of cortisol:cortisone in follicular fluid (assumed to be inversely proportional to the oxidative activity of ovarian 11 β HSD enzymes) is highest when the follicle contains maximal amounts of the hydrophobic 11 β HSD inhibitor(s) and/or minimal amounts of the hydrophilic 11 β HSD stimuli. Conversely, this ratio is lowest in those follicles that contain negligible levels of the hydrophobic 11 β HSD inhibitor(s) and/or high levels of the hydrophilic 11 β HSD stimuli (Thurston et al., 2002b) such that follicular cortisol:cortisone ratios may be determined by the levels of 11 β HSD modulators present within the follicular fluid.

This observation has naturally driven us to ask “Can the levels of 11 β HSD modulators in individual follicular fluid samples predict IVF outcome?” Thus far, this question has been addressed in a double-blind, retrospective study of 127 IVF cycles. Irrespective of IVF outcome, on average, the follicular constituents eluted by 80% methanol have inhibited NADP⁺-dependent cortisol oxidation by a mean value of 61% over 1 hour, and the hydrophilic compounds eluted at 0% methanol have stimulated a mean increase in NADP⁺-dependent 11 β HSD activity of 56%. Cycles characterised by lower levels of the follicular 11 β HSD inhibitors have been associated with a pregnancy rate of 17%, as compared to a pregnancy rate of 46% for those cycles with follicular levels of the ovarian 11 β HSD inhibitors above the outcome-independent average level. Moreover, those follicles with high levels of the hydrophilic 11 β HSD stimuli have been associated with a pregnancy rate of only 10%, whereas the pregnancy rate has been an impressive 59% for cycles in which levels of the ovarian 11 β HSD stimuli have been below average (Thurston et al., 2002a). We propose, therefore, that conception

following gonadotrophin-stimulated IVF-ET may be predicted by high levels of hydrophobic ovarian inhibitors of 11β HSD and/or by low levels of the hydrophilic ovarian stimuli of this enzyme activity.

Our newly acquired data have allowed us to develop a working hypothesis (represented in Figure 3) which can explain why both direct and indirect measurements of ovarian 11β HSD activity can, under defined circumstances, predict IVF outcome. In addition, this model can resolve the questions raised of this biochemical system by some of the negative studies summarised in this chapter.

That the developmental potential of the oocyte and ovarian 11β HSD activities might be independently controlled by a single compound or group of biochemicals is supported by considering the case of glycyrrhetic acid and its hemisuccinate ester, carbenoxolone. These molecules are established inhibitors of 11β HSD derived from liquorice (Duax & Ghosh, 1997; Marandici & Monder, 1993; Stewart et al., 1990) that also influence oocyte maturation by disrupting the gap junctions between the oocyte and the surrounding cumulus cells (Downs, 2001; Webb et al., 2002). However, there is no evidence to suspect that the ability of glycyrrhetic acid and carbenoxolone to close gap junctions is in any way related to their action as inhibitors of the 11β HSD enzymes.

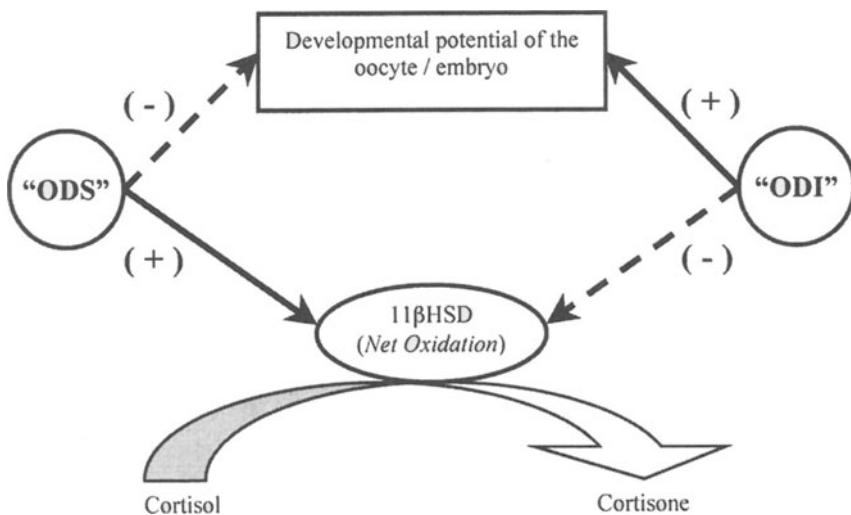


FIGURE 4.3. Working hypothesis for the dual regulation of 11β HSD activities and the developmental fate of the oocyte by paracrine ovarian enzyme modulators.

"ODS" = ovarian dehydrogenase stimulus/stimuli; "ODI" = ovarian dehydrogenase inhibitor(s); (+) indicates a positive effect serving to increase the developmental potential of the oocyte or the net oxidation of cortisol to cortisone; (-) indicates a negative effect serving to impede the developmental potential of the oocyte or inhibit the net oxidation of cortisol to cortisone.

A feature of our working model, under which ovarian 11 β HSD activity would serve as a surrogate biochemical marker for other components of the mature follicle, is that it can explain why targeted deletions of the genes encoding the cloned isoforms of 11 β HSD might not increase pregnancy rates in mice. If it is the levels of enzyme modulators within the ovary (rather than the level of expression of 11 β HSD *per se*) which either determines or reflects the developmental competence of oocytes, decreasing ovarian 11 β HSD activities by a transgenic approach would not mimic the biochemical milieu required in the follicle for optimal development of the oocytes.

Our current model also offers an explanation as to why storage of granulosa cells in follicular fluid for a day or more, and culture for 3 days in serum-supplemented medium may have been necessary for our direct *in vitro* measurements of ovarian 11 β HSD activities to correlate with the probability of conception. The key to this predictive relationship may lie in the extent to which the assay of 11 β HSD activity in the granulosa cells reflects the levels of ovarian enzyme modulators secreted into the follicle by the developing oocyte and/or the granulosa cells. In this event, the relationship between enzyme activities and IVF outcome may be strengthened by storing cells in follicular fluid and/or by culturing the granulosa cells (such that they can continue to produce autocrine/paracrine enzyme modulators) prior to assessment of cortisol metabolism. It seems likely that the logistic considerations which forced us to store granulosa cells prior to transport from Cardiff to London, and the decision to culture cells (to establish a luteinized phenotype) before assaying cortisol metabolism, may have uncovered, by serendipity, a relationship between ovarian enzyme modulators, 11 β HSD activities and IVF outcome.

The presence of modulators of ovarian steroid metabolism in follicular fluid from IVF patients is not without precedent. For example, follicular fluid is known to contain a compound(s) that can inhibit the conversion of androgens to oestrogens by aromatase (Driancourt et al., 1999, 2000; Franchimont et al., 1999). Moreover, increased levels of this compound(s) appear to predict a decreased probability of conception by IVF (Franchimont et al., al., 1999). Follicular fluid also contains compounds that can either enhance or inhibit the synthesis of progesterone by the 3 β -hydroxysteroid dehydrogenase (3 β HSD) enzyme (Girmus & Ledwitz-Rigby, 1987; Kadam et al., 1984). Interestingly, inhibition of 3 β HSD by follicular fluid appears to be mediated (at least in part) by "follicle regulatory peptide" (FRP) (Chicz et al., 1985), levels of which have been associated with successful oocyte fertilization and cleavage *in vitro* (Tonetta et al., 1990). Hence, those compounds that are currently under investigation in the context of the 11 β HSD-IVF relationship may actually belong to a family of biochemical predictors which can modulate the activities of one or more related steroid-

metabolising enzymes. If this proves to be the case, decreased ovarian 11 β HSD activity may simply serve to reflect the follicular levels of enzyme modulators that influence fertility by effecting a pathway of steroid metabolism other than the oxidation of glucocorticoids.

PERSPECTIVES ON BIOCHEMICAL PREDICTORS OF OUTCOME

This chapter has focused on the debate regarding indices of cortisol metabolism as potential biochemical predictors of IVF outcome. In so doing, it has not been possible to consider all of those biochemical markers that have been investigated as predictors of clinical pregnancy rates. Apologies, therefore, to any researcher whose efforts have not been referred to, accepting that a comprehensive overview was not the goal of this chapter. Of the notable omissions, the most prominent is probably the current focus at the University of York on the metabolism of respiratory substrates by oocytes and early pre-implantation embryos (e.g. Downs et al., 2002; Houghton et al., 1996, 2002). This exciting line of research appears to offer the best prospect to date for selecting for replacement those one or two embryos with the highest developmental potential based on a quantitative measure of embryo biochemistry.

In closing, “Do biochemical predictors of IVF outcome exist?” Almost certainly. “Do we know what those predictors are?” Sadly “Not yet!” However, work continues unabated in a number of laboratories to provide a single reliable predictor that can be used to select the best oocytes and embryos and/or to determine the likely outcome of a particular IVF cycle at the earliest opportunity. Sometimes, it appears that these laboratories are in competition, each claiming that their particular parameter is the one that could predict whether a particular cycle is likely to be successful. However, I envisage a situation where more than one lab may be right, not by compromise nor coincidence, but because so many of the parameters that are being considered (ovarian steroids, gonadotrophins, growth factors, cytokines, adrenal steroids and steroid-metabolising enzymes) are inextricably linked in an interdependent manner. As the mist lifts and key predictors emerge, the next challenge will be to establish cause and effect so that we might turn a biochemical marker that *predicts* IVF outcome into a pharmaceutical agent that can be used as an adjunct to *improve* IVF outcome.

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CHAPTER FIVE

GENETICS OF MALE INFERTILITY: EVOLUTION OF THE X AND Y CHROMOSOME AND TRANSMISSION OF MALE INFERTILITY TO FUTURE GENERATIONS

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THE USE OF ICSI IN AZOOSPERMIC AND OLIGOSPERMIC MEN: INTRODUCTION TO THE PROBLEM

Since the introduction in 1992 of intracytoplasmic sperm injection, there has been a revolution in our thinking about male infertility (Palermo et al., 1992; Van Steirteghem et al, 1993). The most severe cases of male infertility, even with apparently 100% abnormal morphology, and even just rare sperm in the ejaculate, could now have pregnancy and delivery rates apparently no different from conventional IVF with normal sperm (Nagy et al, 1995; Liu et al., 1994; Liu et al., 1995). In 1993, testicular sperm extraction (TESE) and microsurgical epididymal sperm aspiration (MESA) in conjunction with ICSI was introduced for the treatment of obstructive azoospermia (Schoysman et al., 1993; Devroey et all, 1994; Silber et al., 1994, 1995a; Tournaye et al., 1994; Devroey et al., 1995a). Eventually this technique was also used for “non-obstructive” azoospermia (Devroey et al., 1995b; Silber et al., 1995b, 1996, 1998a). Many azoospermic men have a minute amount of sperm production in the testis that is not quantitatively sufficient to “spill over” into the ejaculate, but is adequate for ICSI (Silber et al., 1995b, 1995c, 1997a, 1997b; Silber and Rodriguez-Rigau, 1981; Steinberger and Tjioe, 1968; Zukerman et al., 1978). It is with these cases of non-obstructive azoospermia and severe oligospermia that the greatest concern has been registered for the well-being of offspring generated by ICSI. Thus, if severe oligospermia or azoospermia is of genetic origin, in many cases, ICSI creates a potential problem of proliferation of male infertility (Silber 1998b; Faddy et al., 2001).

The purpose of this chapter is firstly to explain the accumulating molecular data on Y chromosomal spermatogenesis genes, and their transmission to

ICSI offspring. The second purpose is to outline the reasons for concentrating on the evolution of the Y chromosome, and the light it sheds on the existence of *many more spermatogenesis genes* that are widespread throughout the genome, and that may also be responsible for transmitting male infertility to future generations. A third, and simpler, goal is to review the more routinely appreciated cytogenetic aspects of male infertility, and its impact on ICSI offspring.

EARLY GENETIC STUDIES OF AZOOSPERMIC AND SEVERELY OLIGOSPERMIC MEN

For several decades, it had been speculated that there was a genetic etiology to many cases of male infertility (Silber et al., 1995b; Silber 1989). This suspicion originally arose from cytogenetic evidence reported over 25 years ago in a very small percentage (0.2%) of azoospermic men who were otherwise phenotypically normal, but who had grossly obvious terminal Y chromosome deletions (Fig. 5.1A,B) (Tiepolo and Zuffardi, 1976).

Simple karyotyping of infertile men also raised the possibility of infertility being associated with autosomal translocations (Van Assche et al., 1996; Bonduelle et al., 1995, 1996, 1998a, 1998b, 1999; Egozcue et al., 2000). A massive summary of karyotyping results in newborn populations, reviewed by Van Assche, revealed an incidence of balanced autosomal translocations in a normal newborn population of 0.25% but an incidence of 1.3%, in infertile men (Table 5.1) (Van Assche et al., 1996). In fact, karyotyping of oligospermic males (i.e. less than 20 million per cc) reveal a 3% incidence of some type of autosomal chromosome anomaly, either balanced Robertsonian translocations, balanced reciprocal translocations, balanced inversions, or extra markers. These translocations could conceivably be transmitted to offspring if ICSI allowed them to conceive. However, because of the limitations of the resolution of cytogenetics, and the very small percentage of these readily discernable karyotypic abnormalities found in infertile men, until recently it had been a convoluted struggle to study the genetic causes of male infertility, and the possible transmission of these genetic errors to the offspring of couples with male infertility (Egozcue et al., 2000).

The possibility that many more cases of male infertility might be genetic was bolstered by the failure of most clinical therapies to correct deficient spermatogenesis (Devroey et al., 1998; Baker et al., 1981; Baker et al., 1984, 1985; Baker and Kovacs, 1985; Baker 1986; Nieschlag et al., 1995, 1998; Nilsson et al., 1979; Rodriguez-Rigau et al., 1978; Schoysman 1983; Silber et al., 1995b; Silber 1989). The heritability of sperm count demonstrated in the wild (O'Brien et al., 1986, 1987; Short 1995), classic studies of naturally occurring pure sterile Y deletions in *Drosophila*, and very early molecular investigations of the Y chromosome in humans led to what has now become an intense search for genes which control spermatogenesis and which may be

defective in many or most infertile males (Johnson et al., 1989; Ma et al., 1992, 1993; Eberhart et al., 1996; Hockstein et al., 1995). However, only recently has the frequent genetic etiology of male infertility related to defects in spermatogenesis (not to mention obstruction) become widely acknowledged via molecular methodology (Kent-First et al., 1996; Kremer et al., 1997, 1998; Krausz and McElreavey, 2001; Silber et al., 1995b; Vogt 1996, 1997; Reijo et al., 1995; Chillon et al., 1995; Shin et al., 1997; Anguiano et al., 1992). If male infertility is of genetic origin, its possible transmission to offspring of successfully treated infertile men is a serious social concern (Page et al., 1999; Mulhall et al., 1998; Silber 1998b; Faddy et al., 2001).

TABLE 5.1 PERCENTAGE OF CHROMOSOME ABNORMALITIES OBSERVED IN SEVEN SERIES OF INFERTILE MEN (AZOOSPERMIC AND OLIGOSPERMIC) COMPARED TO NORMAL NEWBORN POPULATION

All References	Number	Sex Chromosomes	Autosomes	Total
Total	7,876	295 (3.8)	104 (1.3)	399(5.1)
Newborn Infants	94,465	131 (0.14)	232 (0.25)	366 (0.38)

Van Assche et al., 1996

Y CHROMOSOME MAPPING OF INFERTILE MEN AND ICSI

With simple karyotyping, it has been known that a very small number of azoospermic men (0.2%) have large defects visible in the long arm of the Y chromosome that are not present in their fertile fathers. This implied the existence of an azoospermic factor somewhere on Yq. (Tiepolo and Zuffardi, 1976). However, smaller defects (i.e. "microdeletions") could not be discerned with those limited early cytogenetic methods (Fig. 6.1,B). Therefore, these defects in Yq were considered to be rare even in azoospermic men.

In 1992, comprehensive Y chromosomal maps were constructed using yeast artificial chromosomes (YACs) and sequenced tagged sites (STS), and this created the possibility for more detailed study of the Y chromosome in infertile men (Foote et al., 1992; Vollrath et al., 1992). Using polymerase chain reaction (PCR), a more refined search for Y chromosome deletions could be pursued by testing for as many as 52 DNA landmarks (STSs, or sequence tagged sites) across the entirety of the Y chromosome. All Y-DNA markers employed were placed on a physical map of the chromosome, the

markers representing all gene families that were then known in the non-recombining region of the Y chromosome (Vogt et al., 1997; Foote et al., 1992; Vollrath et al., 1992; Lahn and Page, 1997). Using these molecular mapping techniques, which have much greater resolution than cytogenetics, a large series of severely infertile men with clearly identified phenotypes revealed deletions in 13% of azoospermic males (Reijo et al., 1995) (Fig. 5.2).

As many as 7% of severely oligospermic men also had these same "microdeletions" (Silber et al., 1998; Reijo et al., 1996). The most commonly deleted region was located in the distal portion of interval 6, subsequently referred to as AZFc (Fig. 5.3A-C) (Silber et al., 1998; Vogt et al., 1996; Reijo et al., 1995). The higher resolution of Y mapping over karyotyping thus showed that more than just 0.2% of azoospermic men had defects of the Y chromosome, and more than just a few percent of severely infertile men had a genetic cause for their condition. However, because of the highly polymorphic nature of the non-recombining region of the Y (NRY), there are many Y deletions that are of no consequence. Only if these deletions in the

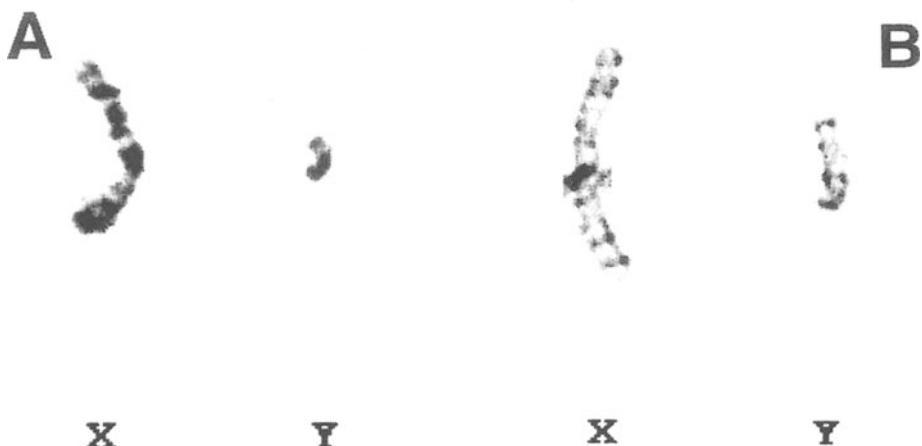


FIGURE 5.1. A AND B . Karyotype of the azoospermic male with cytogenetically visible Yq deletion compared to karyotype of an azoospermic male with a normal Y chromosome.

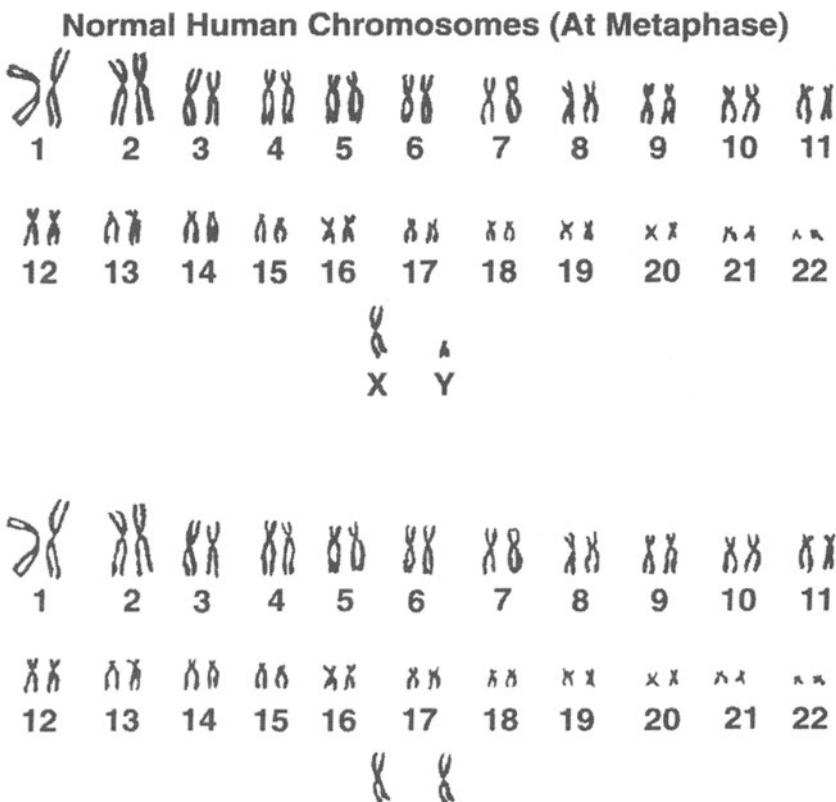


FIGURE 5.1C. Normal male karyotype compared to normal female karyotype.

infertile male are not present in his fertile male relatives, nor in hundreds of normal controls, could they be implicated as a cause of the infertility. The fertile fathers of the Y-deleted, infertile men were shown to have intact Y chromosomes, demonstrating that the deletions had arisen de novo and providing strong evidence that these de novo deletions were indeed the cause of the spermatogenic failure observed in these men. Many laboratories throughout the world have reported on these sub-microscopic deletions of the Y chromosome in azoospermic and severely oligospermic men (Vogt et al., 1996, 1997; Pryor et al., 1997; Ma et al., 1993; Girardi et al., 1997; Mulhall et al., 1997; Kremer et al., 1997; Vereb et al., 1997; van der Ven et all, 1997; Foresta et al., 1997; Chai et al., 1998; Elliot et al., 1997; Nakahori et al., 1996; Qureshi et al., 1996; Najmabadi et al., 1996; Simoni et al., 1997; Bhasin et al., 1994; Kent-First et al., 1996, 1999; Morris and Gleicher, 1996; Krausy and McElreavey, 2001; Chang et al., 1999; Cram et al., 2000; Grimaldi et al., 1998; Kim et al., 1999; Krausyet al., 1999; Liow et al., 1998; Oliva et al., 1998; Seifer et al., 1999; Stuppia et al., 1998; Van Golde et al., 2001;

Van Landuyt et al., 2000; Kremer et al., 1998; Prosser et al., 1996; Van der Ven et al., 1997; Vogt, 1998). Nonetheless, even these popular, new molecular methodologies were crude (not sequence-based) maps, and were suspected of missing huge areas of DNA sequences.

The DAZ gene cluster was identified within the most commonly deleted region, AZFc (Reijo et al., 1995; Saxena et al., 1996) (Fig. 5.A-C). DAZ genes were shown in humans to be transcribed specifically in spermatogonia and early primary spermatocytes (Menke et al., 1997). Autosomal DAZ homologues were also found in *Drosophila* (the Boule gene), in mice (DAZLA), and in fact in frogs and even worms (Table 5.24). These autosomal DAZ gene homologues were found to be necessary for spermatogenesis in every species studied (Eberhart et al., 1996; Cooke et al., 1996; Ruggiu et al., 1997). In the human, Y chromosome DAZ, located in the AZFc region, was found to be in the midst of an area of multiple nucleotide sequence repeats. It was later found to be present in four near identical copies (99.9%) in the AZFc region (Saxena et al., 2000). The presence on human chromosome 3 of DAZLA, an autosomal homologue of the human Y chromosomal DAZ, is what allows a small degree of spermatogenesis to survive in the majority of AZFc-deleted men. However, men with larger deletions that extended beyond AZFc had no sperm at all (Silber et al., 1998). Recently it has been shown that smaller deletions, which take out only two copies of DAZ result in milder spermatogenic defects than the classic AZFc deletion which takes out all four copies of DAZ (deVries et al., 2001, 2002). This indicates that there is a polygenic dosage effect of multiple genes that might control spermatogenesis.

Early ICSI studies showed a clear trend toward larger deletions causing more severe spermatogenic defects than smaller deletions (Silber et al., 1998; Brandell et al., 1998). These studies suggested that possibly several genes in different areas of the Y chromosome might play an important role in spermatogenesis. In fact, some of the earliest studies of deletion on the human Y chromosome unveiled a different gene (RBM) in the AZFb region (Ma et al., 1993; Ma et al., 1992; Kuroda-Kawaguchi et al., 2001; Lahn and Page, 1997; Kobayashi et al., 1994; Elliott et al., 1997). Although there are numerous copies and pseudogenes of RBM on the Y, most of which are nonfunctional, there appears to be a functional copy in the region just proximal to AZFc, with no “rescue” homologues elsewhere. These early results supported the concept, that numerous genes on the Y chromosome, in addition to those of the AZFc region, impinge on spermatogenesis (Lahn and Page, 1997). As might have been expected, many more genes have now been identified in AZFc and elsewhere on the Y by detailed sequencing studies (Lahn and Page, 1997, 1999a; Kuroda-Kawaguchi et al., 2001). Thus, multiple spermatogenesis genes apparently contribute to and modify the severity of the spermatogenic defect in Y-deleted men.

These early deletions on the Y were called “micro” deletions only because they could not be discerned by karyotyping. But they were indeed huge deletions (Kuroda-Kawaguchi et al., 2001). It was correctly hypothesized that smaller deletions, or point mutations might very well be present both in AZFc and elsewhere, but the repetitive nucleotide sequences which characterize much of the Y chromosome made it very difficult with standard STS markers to define smaller deletions (Sun et al., 1999).

The unusually repetitive sequence structure of the AZFc region of the Y plagued even the first attempts at constructing a physical map with YAC’s, because repetitive STS’s could not be accurately placed in what was then called deletion intervals 6D-6F. Even the size of AZFc (without an accurate sequence) was controversial (0.5 to 2 Mb) (Foote et al., 1992; Yen 1998; Tilford et al., 2001). Efforts to find point mutations along the Y chromosome, have also been thwarted by the presence of multiple copies of genes in these regions with numerous Y specific repeats that in the absence of a complete sequence made the detection of specific nucleotide errors almost impossible to detect. The Y chromosome, and specifically the most commonly deleted area, such as AZFc, defied sequencing by the usual methods. Therefore, the AZFa section of the Y was initially selected to study in detail, because of the apparent absence of multiple gene copies or Y-specific repeats in that region (Sun et al., 1999, 2000).

AZFa, has a completely different, more conventional and non-repetitive structure than AZFb or AZFc, making this much less commonly deleted region of the Y an ideal starting-off point. Therefore, the AZFa region of the Y chromosome was the first region of the Y to be sequenced, and two functional genes were identified, USP9Y and DBY (Sun et al., 1999; Sun et al., 2000). Sequencing of these two genes in 576 infertile men and 96 fertile men revealed several sequence variants most of which were inherited from the fertile father and of no functional consequence. However, in one case a de novo point mutation was found on USP9Y (a four base pair deletion in a splice-donor site, causing an exon to be skipped and protein truncation). This mutation was absent in fertile relatives and represented the first case of a point mutation causing a single gene defect associated with spermatogenic failure. This particular region of the Y was more amenable to such a mutation search because of the lack of sequence repeats which plague the rest of the Y chromosome. This finding offered a hint at what we might find if we were able to search for more subtle gene defects in the larger areas of the Y chromosome where most of the testis specific genes have been located (Lahn and Page, 1997; Kuroda-Kawaguchi et al., 2001).

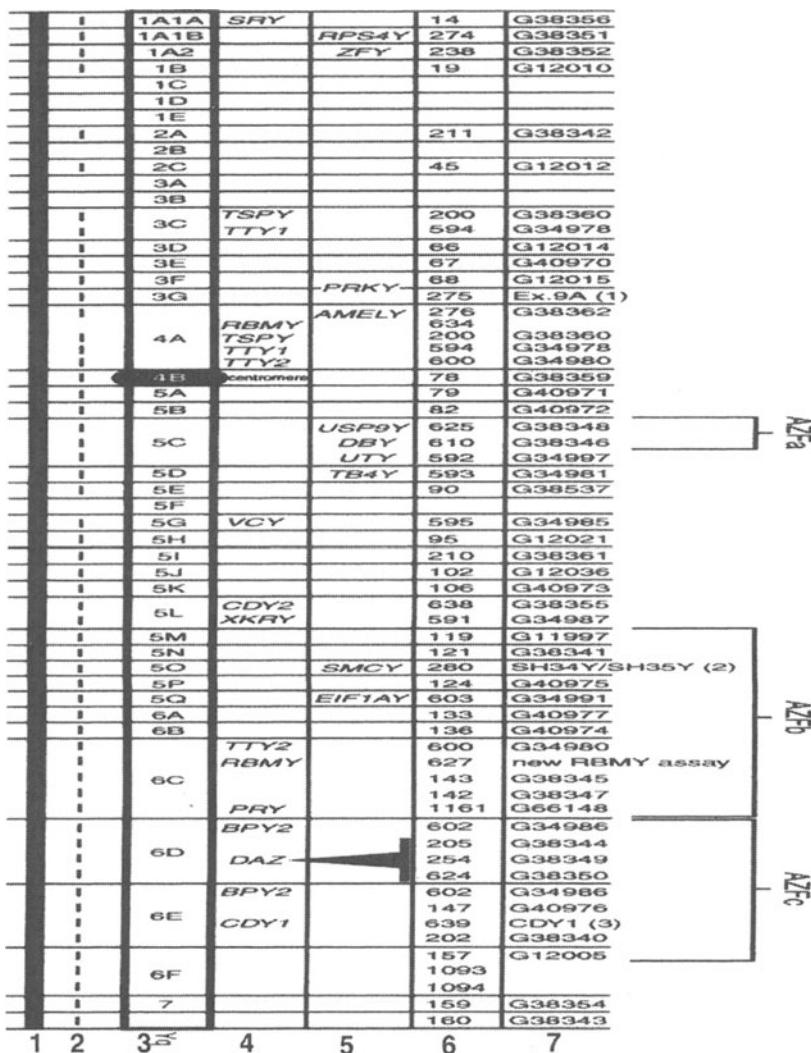


FIGURE 5.2. Y chromosome map based on STS interval markers and their corresponding X-homologous and testis-specific gene. 1. Fertile male control; 2. Fertile female control; 3. Yp; 4. Testis-specific gene families; 5. X-homologous genes; 6. SY#; 1. GenBank STS accession number (or ref.).

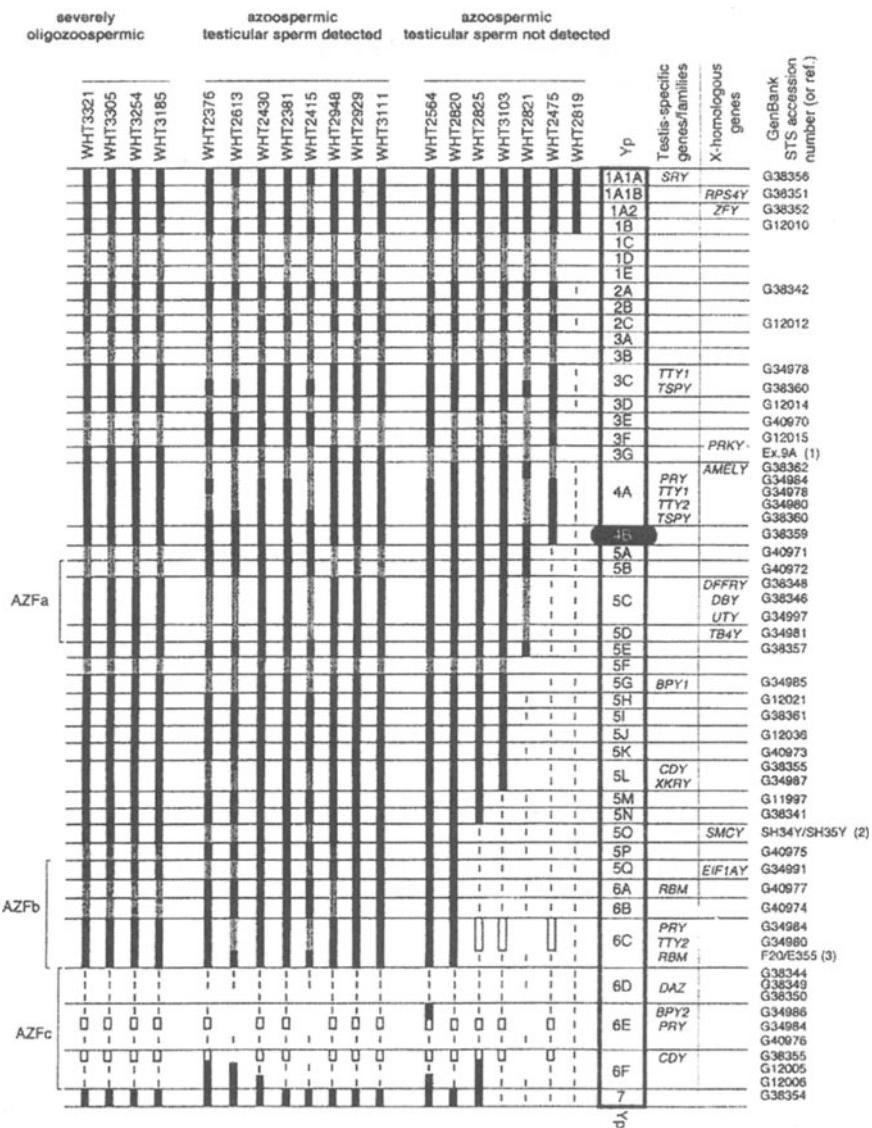


FIGURE 5.3A. Typical early deletion map of azoospermic and severely oligospermic men with chromosomal microdeletion.

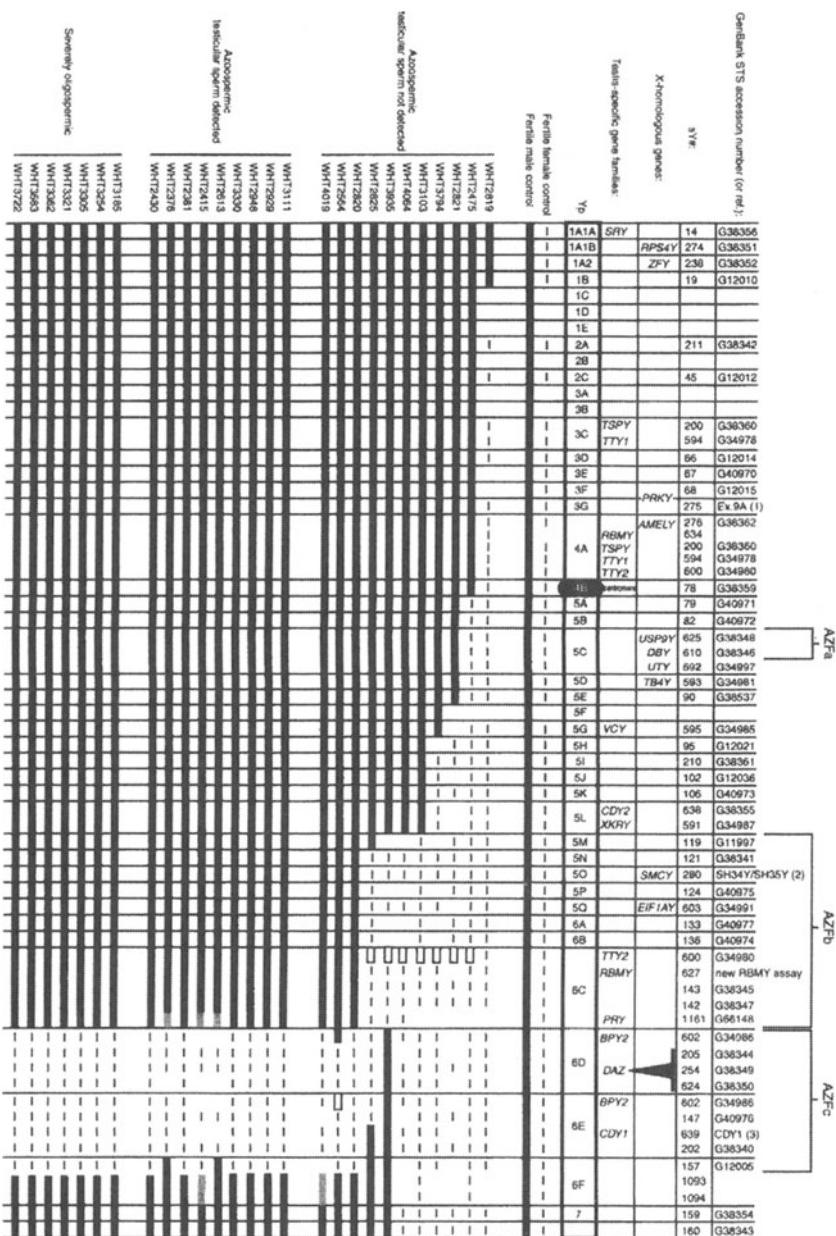


FIGURE 5.3B.

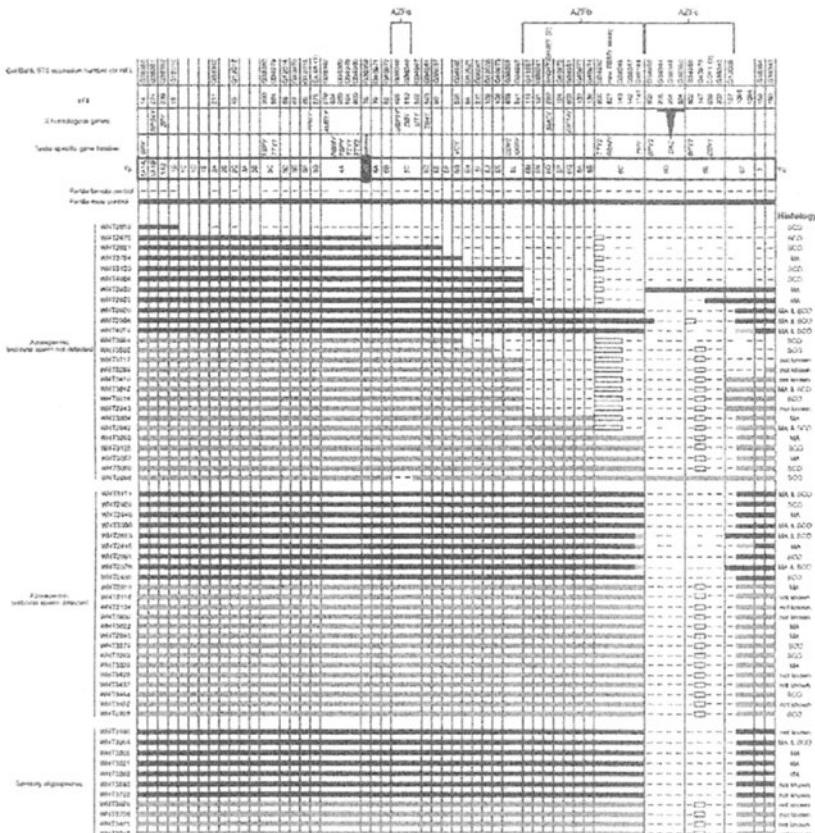
USP9Y. This finding in the AZFa region runs parallel to previous observations that larger Y deletions (which take out more genes) are associated with a lesser likelihood of finding sufficient sperm for ICSI (Silber et al., 1998).

TABLE 5.2 GENES OF THE DAZ FAMILY IN VERTEBRATES AND DROSOPHILA

	Y CHROMOSOMAL	AUTOSOMAL
Human	DAZ cluster (at least	DAZL
	4 copies, > 99% identical	(chrom. 3)
Mouse		DAZL (Dazla)
		(chrom. 17)
Xenopus		Xdazl
Drosophila		boule
C. elegans		daz-1

Shortly after we began our Y chromosomal mapping study of infertile men, intracytoplasmic sperm injection (ICSI) with testicular and epididymal sperm retrieval methods for azoospermia were developed (Schoysman et al., 1993; Devroey et al., 1994; Silber et al., 1994, 1995a; Devroey et all, 1995a; Silber et al., 1995b, 1996; Silber 1998a; Silber et al., 1995; Tournaye et al., 1994; Mulhall et al., 1997). Men with the most severe spermatogenic defects causing azoospermia in the ejaculate could now have children. Thus, at the very moment in time that we had an effective treatment for severe male infertility, the reality that male infertility is often of genetic origin, also became generally recognized. Subsequently it was demonstrated that these Y deletions would be transmitted to offspring as a result of ICSI (Silber et al., 1998; Silber 1998a, 1998b; Page et al., 1999). When sperm were recoverable in azoospermic or oligospermic men, there was no significant difference in fertilization or pregnancy rate with ICSI whether the man was Y-deleted or not (Table 5.3 and 5.4). Large defects resulted in complete azoospermia but smaller defects were associated with the recovery of some sperm sufficient for ICSI, and even occasionally spontaneous offspring as well (Silber et al., 2001).

Y Deletions and Diverse Histology in Azoospermic and Severely Oligospermic Men



The experimentally demonstrated presence of a locus in an individual is indicated by a black segment; the gray segment remains "presumably" not repeated and no DNA sequencing is test; experimentally demonstrated absence is indicated by a minus, and inferred absence is indicated by the absence of any symbol. White boxes represent positive PCR results, and these must be interpreted as the result of the Y-STRs; recent entries of the sequences being considered. It is very likely that these positive results reflect the existence of closely related, cross-amplifying sequences in other portions of the Y chromosome.

FIGURE 5.3B AND 3C. More refined, later deletion maps of azoospermic and severely oligospermic men.

Studying AZFa also provided a good model for the interaction and overlapping functions of multiple genes which sheds light on the “polygenic” nature of the genetic control of spermatogenesis. When the entire AZFa region is deleted, taking out both DBY and USP9Y, there is a more severe spermatogenic defect and the patient is azoospermic. However, when there is only a specific point mutation of the USP9Y gene, we observed maturation arrest with a few pachytene spermatocytes developing into mature sperm in a few seminiferous tubules. Thus, the loss of DBY (the only other gene in the AZFa region) exacerbates the spermatogenic consequences of the loss of

TABLE 5.3 RESULTS OF ICSI IN Y-DELETED VERSUS Y NON-DELETED MEN WITH SEVERE OLIGOSPERMIA ($<2 \times 10^6$) AND AZOOSPERMIA (NON- OBSTRUCTIVE)

	Y-Deleted ($<2 \times 10^6$)	Not Y-Deleted ($<2 \times 10^6$)
N. Patients (with sperm)	23	205
N. Cycles (with sperm)	45	312
N. Eggs	508	3291
N. 2PN	(289) 57%	(1849) 56%
N. Pregnant	(17) 38%	(112) 36%
N. Deliveries	(13) 29%	(81) 26%
N. Babies	18	99
N. Boys	10	43
N. Girls	8	56

Silber, Oates, Brown, Page (2001)

TABLE 5.4 Y DELETION DETECTION IN PERIPHERAL LYMPHOCYTES OF 884 INFERTILE MEN (S/A $<5 \times 10^6$)

Diagnosis	All Y Deletions	
	Number Studied	Y Deletion Found
Non-obstructive azoospermia	528	66(13%)
Severe oligospermia ($<5 \times 10^6$)	356	24 (7%)
Totals	884	90 (10%)

As of December 2001

WHY THE "Y"?

Why should the initial molecular efforts at defining the genetic causes of male infertility have concentrated on this difficult Y chromosome with all of its confounding repeats, polymorphisms, and degenerating regions? The answer lies in the evolutionary history of the X and Y chromosome. Over the course of the last 240-320 million years of mammalian evolution, the X and

the Y chromosome have evolved from what was originally a pair of ordinary autosomes (Fig. 5.4) (Lahn and Page, 1997, 1999a; Rice 1992, 1994, 1996; Graves 1995a, 1995b, 2000). During that evolution, just as most of the ancestral X genes were decaying because of the lack of meiotic recombination of the developing X and Y chromosomes, genes which control spermatogenesis arrived (by transposition or retroposition) from autosomes to the Y (Fig. 5.5). Once on the Y, these formerly autosomal genes amplified into multiple copies, and achieved greater prominence (Saxena et al., 1996; Lahn and Page, 1999a). Spermatogenesis genes that arrived on the Y, but came originally from autosomes, include the well-studied DAZ and CDY (Saxena et al., 1996, 2000; Lahn and Page, 1999b) (Fig. 5.5). Other spermatogenesis genes on the Y have persisted from their original position on the X and developed specific spermatogenic function on the Y, and also into numerous copies on the Y, such as RBM (Delbridge et al., 1997; Vogel et al., 1999; Delbridge 1999a, 1999b; Mazeyrat et al., 1999).

Classical Model of Sex Chromosome Evolution: Y as Decayed X

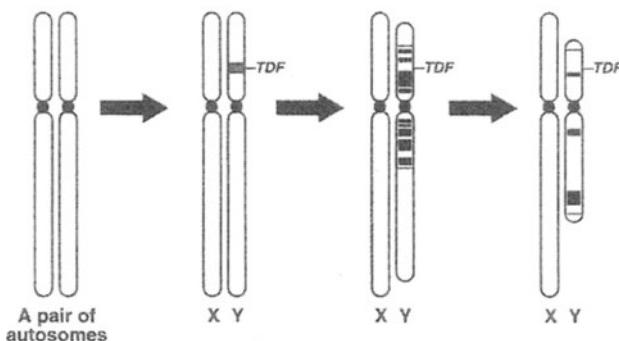


FIGURE 5.4. Figurative outline of evolutionary degeneration of one chromosome with the testicular determining factor (TDF) gene which doesn't recombine with its homologue, resulting eventually in a Y chromosome.

Although DAZ is a very ancient, well conserved gene, readily found to be functional in autosomes of *c. elegans* (worms), *drosophila* (fruit flies), *xenopus* (frogs), and rodents, it is only found on the Y chromosome of old world monkeys, apes, and humans (Table 5.2). In earlier mammals and in non-mammalian species, it is otherwise purely autosomal. RBM, however, is found on the mammalian Y as far back as the Y's origin, as evidenced by its presence on the Y of marsupials even before the divergence of eutherian from non-eutherian mammals. Thus, RBM was a spermatogenesis gene which

began on the ancestral autosomes that evolved into the mammalian X and Y chromosomes. The ancestral RBM that remained on the X chromosome (RBMX) retained its “widespread” function, whereas RBM-Y, which persisted on the receding Y chromosome, evolved a male-specific function in spermatogenesis (Delbridge et al., 1999; Mazeyrat et al., 1999; Graves, 1997; Pask et al., 1999).

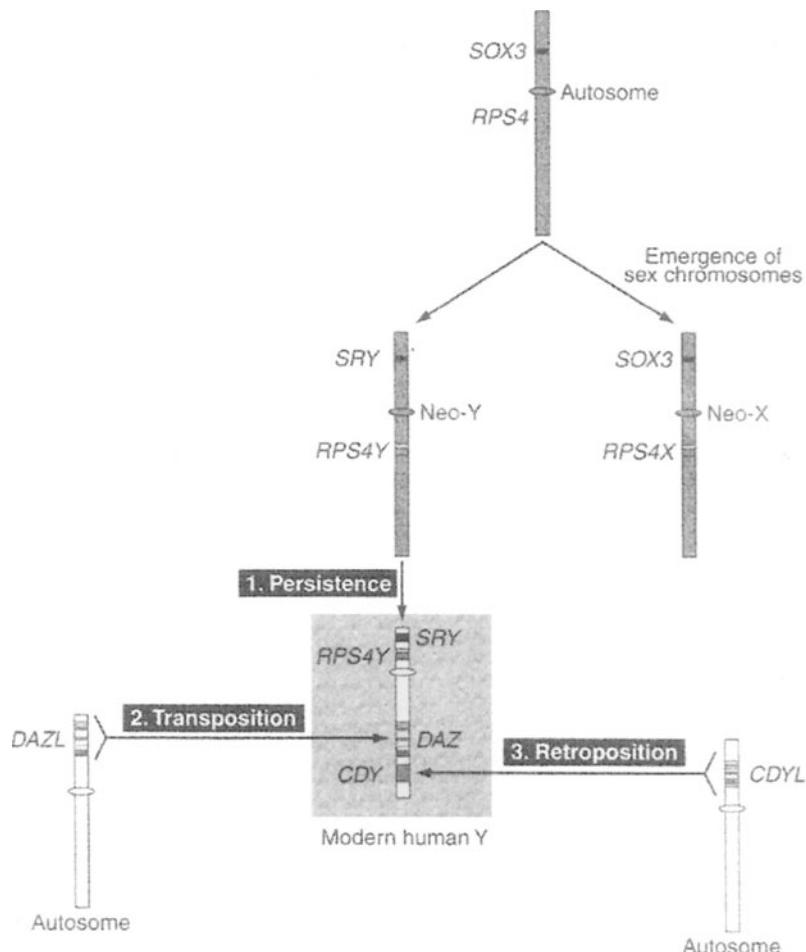


FIGURE 5.5. Over the course of evolution, the Y chromosome descended from the ancestral autosome that developed the SRY male-determining gene. The Y then attracted male-specific genes by three mechanisms (Lahn and Page modified, 1999b).

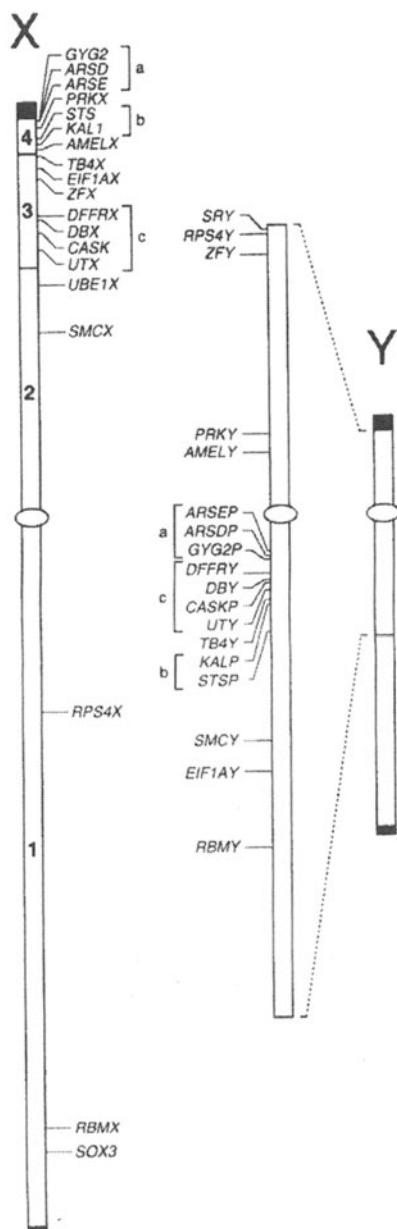


FIGURE 5.6. Graphic depiction of X-homologous genes on the Y chromosome representing four different stages of a divergence from its original ancestral X showing corresponding X and Y homologous genes. Note that SOX3 and RBMX come from the earliest region of sequence divergence and correlate with the SRY gene and the RBMY gene. (Lahn and Page, 1999a).

Indeed, even the SRY gene (the male sex-determining locus) was probably originally the SOX3 gene on the ancestral X prior to differentiating into the SRY male sex-determining gene. In fact, the evolution of a non-recombinant male determining gene (SRY) is what actually began the whole process of the Y chromosome's evolution. SOX-3 is a gene on the X chromosome which inhibits SOX-9 also on the X chromosome. SOX-9 (on the X chromosome) is the gene that actually activates male sex determination. SOX-3 evolved into SRY on the ancestral Y chromosome. SRY inhibits SOX-3 from suppressing SOX-9, and thus determines whether the SOX-9 cascade of events leading to the formation of a testis takes place. That was the beginning of the transformation of an ordinary pair of autosomes into the modern X and Y. (Fig. 5.6) (Lahn and Page, 1999b; Graves 1997, 1995a, 1995b; Vidal et al., 2001).

Genes associated with the non-recombinant SRY region that were specifically beneficial for male function or antagonistic to female function, flourished on the evolving Y chromosome because it was a “safe harbor,” without the detrimental effect of meiotic recombination which would have otherwise allowed male-specific genes to be expressed in females (Lahn and Page, 1997, 1999a, 1999b; Silber 1999). In this way, “male benefit” genes have arrived and accumulated on the evolving Y chromosome over many millions of years via the three mechanisms of: “transposition” from an autosome via translocation, “retroposition” from an autosome via reverse transcription, and “persistence,” i.e., male modification of function from what was originally a gene on the ancestral X. This process gives the Y chromosome a very unique type of “functional coherence” not seen elsewhere in the human genome (Fig. 5.7) (Lahn and Page, 1997). However, like with SRY, we should not be surprised to find that many genes which are male-specific could be on the X as well, and sprinkled throughout the genome.

Two Gene Classes Reflect Sequence Organization of NRY

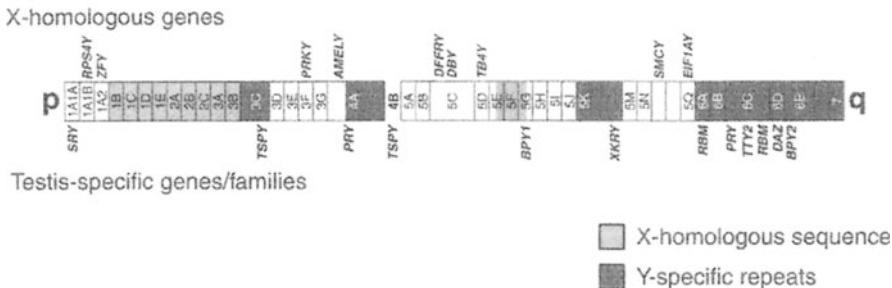


FIGURE 5.7. Y chromosome has a remarkable functional coherence not seen in any other chromosomes. Genes depicted above are X-homologous because of their equal similarity to genes on the X chromosome. Genes depicted below are Y-specific genes which are testis specific, expressed only in the testis, and have no X-homologues. (Lahn and Page, modified, 1997).

FUNCTIONAL REPRODUCTIVE ANATOMY OF THE X AND Y CHROMOSOME

Translocations occur on a relatively frequent basis in any species. Over evolutionary time, this results in conserved, homologous genes of different species residing in completely different parts of the genome and in a relatively mixed-up array of genes in every chromosome, where structural proximity has little or no relationship to function (Lahn and Page, 1997). However, these random transpositions (which over the course of time result in a chaotic lack of apparent organization of the genome) have also allowed direct acquisition by the Y of genes that have a common function to enhance male fertility. Selective pressures favor the process of spermatogenesis genes concentrating on the non-recombining portion of the Y chromosome in association with the male sex-determining gene, SRY, particularly if these genes are of little benefit to females or actually diminish “female fitness” (Saxena et al., 1996; Rice 1992, 1994, 1996; Silber 1999; Graves 1995; Winge 1927; Charlesworth and Charlesworth, 1980; Hackstein and Hochstenback, 1995).

Quite interestingly, the X chromosome, unlike autosomes, and unlike the Y chromosome, has been remarkably conserved in all mammals, with little mixing of genes from elsewhere. This is because of the selection against

disruption of development of the X-inactivation process in the evolution of the X and Y (Graves et al., 1998)

Genes which arrived on the Y, or which persisted on the degenerating Y from the ancestral X, and gained prominence on the Y, underwent paradoxical processes of amplification, producing multiple copies, and degeneration because of the failure of recombination. DAZ (as has been discussed) was the first such gene which was identified in the AZFc region of the Y chromosome by our initial Y mapping in azoospermic men (Reijo et al., 1995). DAZ represents the first unambiguous example of autosome-to-Y transposition of a spermatogenesis gene, which is representative of a generalized process that effects many other spermatogenesis genes, and indeed possibly explains the relatively poor state of affairs of human spermatogenesis compared to that of other animals (Saxena et al., 1996; Silber 1999). Autosome-to-Y transposition of male fertility genes appears to be a recurrent theme in Y chromosome evolution throughout all species. The autosomal DAZ gene (in humans called DAZ-L) is located on human chromosome 3, and on mouse chromosome 17. At some point during the evolution from new world to old world monkey, about 30 million years ago, this DAZ gene arrived on the Y by transposition from what is now human chromosome 3, and there multiplied to produce four almost identical gene copies. This process was first depicted for DAZL and DAZ. However, there are now known to be other previously autosomal genes or gene families on the Y that are expressed specifically in the testis, and are also likely to play a major role in spermatogenesis (Table 5.5) (Lahn and Page, 1997).

The CDY gene arrived on the AZFc region of the Y chromosome in a different fashion than DAZ, via reverse transcription (Lahn and Page, 1999a). The autosomal CDY gene (CDY-L) is located on mouse chromosome 13 and on human chromosome 6. CDY's intron-free homologue found its way to the human Y actually prior to the arrival of DAZ, sometime after the prosimian line of primates separated off, approximately 50 million years ago (Fig. 5.8). It did so by reverse transcription and, therefore, has very few introns in marked contrast to CDYL, its autosomal homologue on chromosome 6, which is intron-rich (Lahn and Page, 1999a).

TABLE 5.5 ARRIVAL OF SPERMATOGENESIS GENES TO Y CHROMOSOME

	Y Gene	Ancestral Gene
Transposition	DAZ (AZFc)	DAZL (autosomal)
	Human-Y	Mouse 17 Human 3
Retroposition	CDY (AZFc)	CDYL (autosomal)
	Human-Y	Mouse 13 Human 6
Persistence	RBM (AZFb)	RBMX
	Human-Y and Mouse-Y	Mouse X Human X
Persistence	SRY	SOX-3

The RBM gene, on the AZFb region of the Y chromosome, had its origin in our ancestral X chromosome, and there it amplified and gained prominence as a testis-specific gene. 240 to 320 million years ago, shortly after the divergence of the mammalian and avian lineages, the X and Y began to diverge in sequence identity with the emergence of SRY and the failure of recombination in the region of SRY (Fig. 5.9) (Lahn and Page, 1999b).

As the evolving Y chromosome underwent decay because of lack of recombination, these genes (which were originally X chromosomal) diverged in sequence on the Y, and those which had “male benefit” functions persisted (Table 5.6). The prime example of such genes, of course, is the SRY gene itself, that began as the generic SOX3 on the X chromosome, but then developed its specialized “testis determining function,” originating the whole process of the non-recombination, which resulted ultimately in degeneration of the Y chromosome (Lahn and Page, 1999b). This same process is how RBM arrived to prominence on the AZFb region of the Y (Lahn and Page, 1999b; Vogel et al., 1999; Delbridge et al., 1999a, 1999b; Mazeyrat et al., 1999; Graves 1995; Cooke et al., 1996).

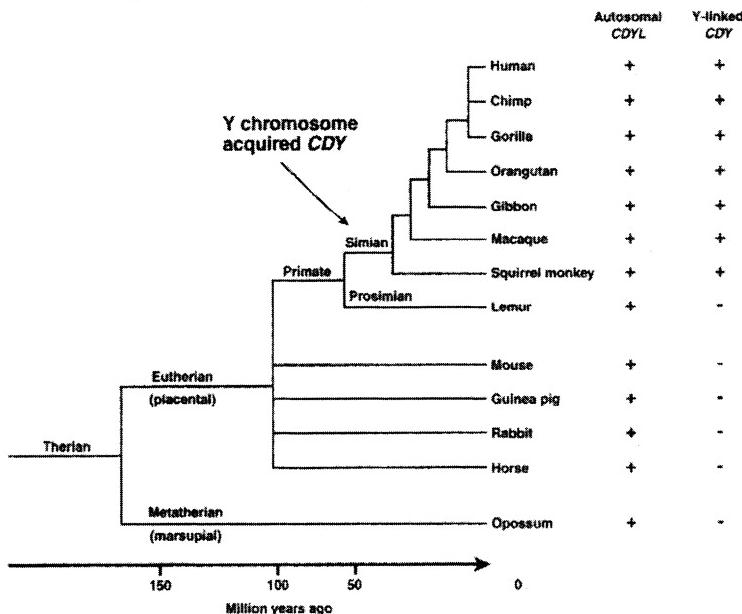


FIGURE 5.8. Whereas DAZ was transposed to the Y chromosome approximately 30 million years ago (after the divergence of old world and new world monkeys), CDY arrived on the Y chromosome much earlier (50 million years ago) by a process of reverse transcription. (Lahn and Page, 1999b).

The AZFa region of the NRY is a little more complicated. As the ancestral Y began to recede in comparison to its paired X, it did so in stages and “strata” over about 320 million years (Fig. 5.9). There are four clearly definable strata on the X chromosome that decrease in X-Y homology

according to how early in their history they failed to recombine (Lahn and Page, 1999a). As a given stratum of the X failed to recombine with its Y counterpart, homologous X-Y genes in that stratum diverged in sequence structure (Fig. 5.6). The most recent areas of non-recombination of X genes is located most proximally on the X and the most ancient areas of non-recombination are located most distally on the X. The AZFa region of the Y chromosome diverged from the X fairly recently in its evolutionary history and, therefore, has a much more conventional sequence structure, with much greater homology to its counterpart on the X. The two genes in AZFa (USP9Y and DBY) both play an important role in spermatogenesis, in that deletion of AZFa results in a complete absence of sperm. Yet they have very close homologues on the X, and are still ubiquitously transcribed (Sun et al., 1999, 2000).

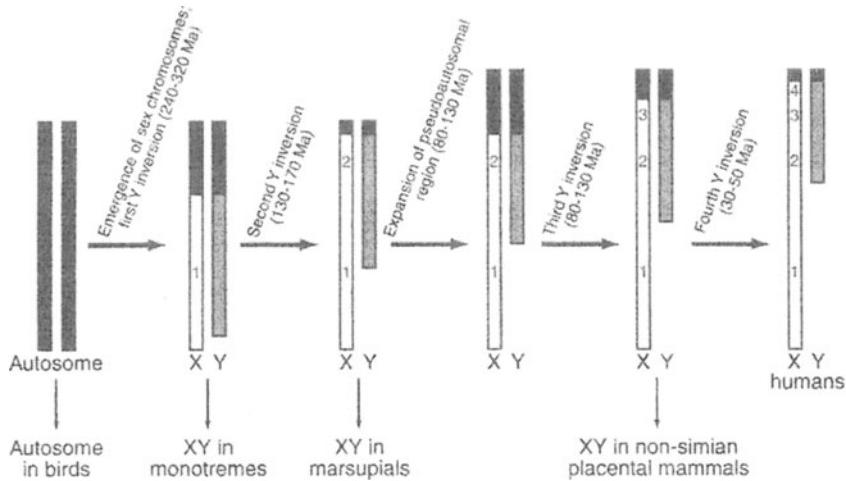


FIGURE 5.9. The X and the Y chromosome develop in mammals at the time of divergence in the avian and mammalian line by a series of four well defined inversions. The earliest inversions (region 1 on the X) have the least similarity to their Y-homologue, and genes in the most recent area of divergence (region 4) have the greatest sequence similarity of their counterpart on the Y. (Lahn and Page, modified, 1999a).

Regardless of the method of arrival of spermatogenesis genes to the non-recombining portion of the Y, this region would inevitably face, and likely succumb, to powerful degenerative forces during subsequent evolution (Saxena et al., 1996). Saxena postulated that, “perhaps the rate of acquisition of male fertility genes approximates the rate of subsequent degeneration, resulting in an evolutionary steady state. In contrast to the extreme evolutionary stability of the X chromosome, at least in mammals, individual

male infertility genes might not be long lived, in an evolutionary sense, on the Y chromosome."

Our emphasis on the Y chromosome for locating spermatogenesis genes to help in elucidating the causes of male infertility makes sense, because the Y has collected for us genes that otherwise would be hidden throughout the genome. However, it would be naïve to assume, in view of the evolutionary history of the X and the Y, that there are not equally powerful components for regulating spermatogenesis located also on the X chromosome and on the autosomes. Some have speculated that the instability of the Y chromosome may lead to an inexorable decline in sperm production in the evolution of any species, unless there is either sperm competition within the mating pattern of the species, or a method of continual recruitment of new spermatogenesis genes to the Y chromosome with subsequent amplification prior to ultimate degeneration (Silber 1999). The Y chromosome is a favorable place to begin a molecular search for genes that affect male fertility. But the very reason for starting with the Y emphasizes the likelihood of finding more such genes hiding throughout the genome.

TABLE 5.6 PERSISTENCE ON Y OF RBM X

Y Gene	Ancestral Gene
SRY	SOX-3
Determines Male Sex	No male specific function
RBM-Y	RBM-X
MALE SPECIFIC FUNCTION	NO MALE SPECIFIC FUNCTION
Numerous copies	One copy
Many degenerate	
Same in all etherian mammals	

PARALLEL AND INDEPENDENT EVOLUTION OF X AND Y CHROMOSOMES IN HUMANS AND ANIMAL MODELS: THE ORIGIN OF X-INACTIVATION (E.G., WORMS, FLIES, EVEN FISH)

Sex chromosomes have evolved independently many times in different genera with the same common theme. The chromosome with the sex-determining gene progressively loses the ability to recombine with its mate, accumulates mutations, and embarks on an inexorable deterioration. For example, the mammalian Y chromosome and the Drosophila Y chromosome (not to mention the ZW system in avians) have nothing in common with each other except their name, and the fact that they do not recombine with their larger counterpart, which is called the X chromosome. The X and Y

chromosomes evolved completely separately and differently in each of these well studied groups of species, but remarkably they evolved via the same common evolutionary theme.

If the Y chromosome of *Drosophila* has a deletion, the *Drosophila* is sterile. If the Y chromosome of the mouse or human has a deletion, the mouse or human is sterile. In any species thus far studied, if the Y chromosome has a significant deletion, that species is sterile. However, the genes that would have been deleted on the *Drosophila* Y, or the mouse Y chromosomes, are not the same genes that are deleted in the human Y. For example, the homologue of the human Y DAZ gene on *Drosophila* is autosomal, (the so-called “boule” gene), just as it is also autosomal in the mouse (DAZLA), and the deletion of this autosomal gene in *Drosophila*, or in the mouse, results in sterility just as readily as deletion of the *Drosophila* Y or the mouse Y chromosome (Cooke et al., 1996; Eberhart et al., 1996; Ruggiu et al., 1997). Deletion of the DAZ genes on the Y chromosome of humans often does not result in complete absence of spermatogenesis, possibly because the ancient DAZ autosomal homologue on chromosome 3, rescues spermatogenesis to some small extent. Deletion of AZFb genes, however, usually result in total absence of sperm, probably because there are no effective autosomal or X homologues to rescue spermatogenesis when these genes are deleted.

The same pattern is found in all species studied. The X and Y begin as a pair of ancestral autosomes in which a male-determining gene (which does not recombine with its homologue) begins the inexorable process of decay into what then becomes a Y chromosome. In some *Drosophila*, the Y chromosome has disappeared altogether, and the resultant XO male is sterile. Although the human Y chromosome (or for that matter, any of the mammalian Y chromosomes) has no nucleotide sequence similarity at all to the fruit fly’s Y chromosome, the same mechanism of accumulation of spermatogenesis genes to a decaying male sex determining chromosome is operating (Silber, 1999). Thus, the Y chromosome of the *Drosophila*, and the mouse, is quite different than the Y chromosome of the human, but yet they appear to be the same because of the common two evolutionary themes in the development of the Y. One theme is its gradual decay from what was its autosomal homologue, but is now the X chromosome, and the second theme is its growth from acquisition and accumulation of male benefit specific genes from other parts of the genome.

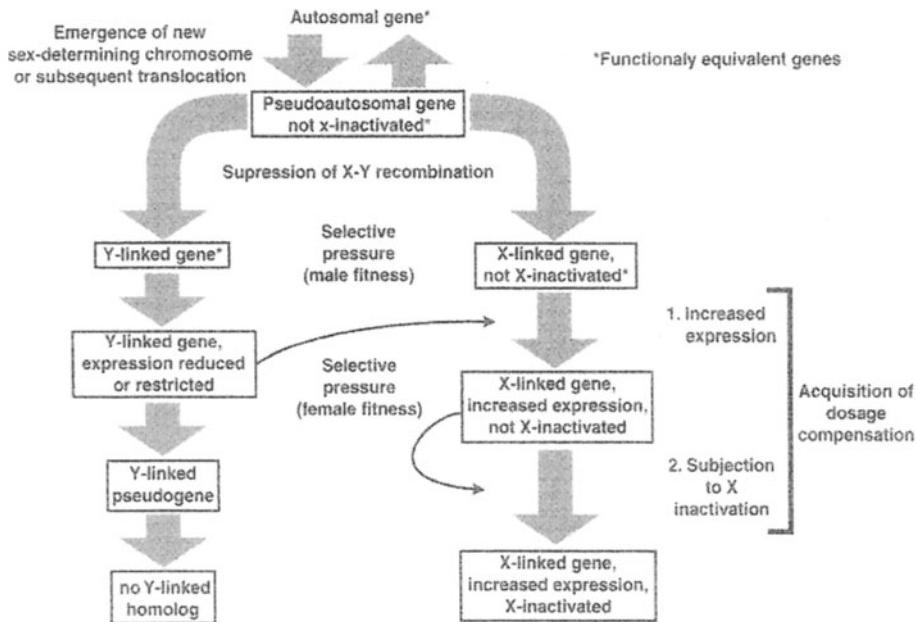
This evolutionary mechanism of degeneration of the Y, and accumulation of spermatogenesis genes may explain the relatively high frequency of male infertility and poor sperm quality in species (like ours) that have minimal sperm competition. It may also explain the phenomenon of X inactivation, the high frequency of XO human stillbirths, and the survival of some XO concepti as Turner’s Syndrome children.

The ancestral autosome which is to become the X chromosome develops a process first of hyperactivation, and then X inactivation, to make up for the decay of homologous alleles on what is now becoming the Y chromosome (Jegalian and Page, 1998; Graves et al., 1998). As the X retained, and the Y gradually lost, most of these ancestral genes, expression of the X had to, at first, be increased to compensate for the male's loss of these genes, and X inactivation had to develop in the female for X genes whose Y homologue had eventually disappeared (Fig 5.10). The problem of X chromosome dosage differences in males and females is solved by inactivation in the female of one of the two X chromosomes combined with upregulation of the remaining X chromosome in females and the single X chromosome of males. This mechanism also insured the remarkable conservation and similarity of the X chromosome in all mammalian species.

An understanding of the evolution of X-homologous Y genes losing their general cellular functions, requiring upregulation, and inactivation of X genes on one of the two female X chromosomes, helps to clarify the different stages of evolutionary development of the mouse and human Y. The RBMY gene is a testis-specific male benefit spermatogenesis candidate gene. RBM's homologue on the X (RBMX) developed no male specific expression, but retained its general cellular housekeeping function. Thus, RBMX would be expected to behave like an X gene with no Y counterpart and probably undergo X inactivation (Table 5.7). As a related example, human ZFX and ZFY genes are very closely related homologues on the X and Y chromosome, both of which have general cellular housekeeping functions that are critical for life. Therefore, ZFX escapes X-inactivation in the female and ZFY is, therefore, probably one of the Turner genes (Lahn and Page, 1997; Jegalian and Page, 1998). However, the mouse is quite different. In the mouse, ZFY appears to have evolved a male-specific function, and, therefore, ZFX in the mouse has a general housekeeping function not shared with its Y homologue. Thus, ZFX in the mouse is X-inactivated, even though in the human it is not.

Similarly, RPS4X and RPS4Y are another homologous pair of genes on the X and Y, both of which have equivalent housekeeping functions in the human. Therefore, in the human RPS4X, similarly to ZFX, is not subject to X-inactivation because there are functional transcripts in men, from the X and Y, and in women from the two X's. In mice, however, RPS4Y has not only lost its function in evolution, but has degenerated out of existence. Therefore, in the mouse RPS4X is subject to X-inactivation, just as most of the *genes on the X chromosome in all animals require X-inactivation if they don't have a functioning homologue on the Y*.

This summary of the evolutionary history of our X and Y chromosome explains why the Y chromosome was a good place to start in our molecular search for spermatogenesis genes. However, it is clear that numerous genes from throughout the genome, though less well studied, also impinge on spermatogenesis, and may thus be transmitted to ICSI offspring.



Jegalian & Page, *Nature* 394:776 (1998)

FIGURE 5.10. X-inactivation develops after X-linked hyperexpression as a pairing mechanism between the evolving X and Y chromosomes to compensate for decay of X genes on the evolving Y. (Jegalian and Page, 1998).

KARYOTYPE OF INFERTILE MALES AND OF ICSI OFFSPRING

The incidence of cytogenetically recognizable chromosomal abnormalities in the offspring of ICSI patients is acceptably very low, but much greater than what would be anticipated in a normal newborn population. Follow-up of the first 1,987 children born as a result of ICSI has been meticulously studied and reported by the originators of ICSI in the Dutch-Speaking Free University of Brussels (Bonduelle et al., 1995, 1996, 1998a, 1998b, 1999). In 1,082 karyotypes of ICSI pregnancies, 9 (0.83%) had sex chromosomal abnormalities, including 45,X (Turners), 47, XXY (Klinefelter's), 47, XXX and mosaics of 47, XXX, as well as 47, XYY (Table 5.8). This is a very low frequency of sex chromosomal abnormalities, but nonetheless is four times greater than the expected frequency of sex chromosomal abnormalities in a newborn population (0.19%). Obviously the 45,X and 47,XXY children will be infertile (0.5%). Four (0.36%) of the 1,082 offspring had de novo balanced autosomal translocations or inversions. These children were apparently normal, but this incidence of de novo balanced autosomal translocations is

five times greater than what would be anticipated in a normal newborn population (0.07%), and these children might also be suspected of growing up to be infertile (0.36%).

TABLE 5.7 PERSISTENCE ON Y OF X GENES

	Y Gene	Ancestral X Gene
	SRY	SOX-3
	Determines male sex	No male specific function
	RPS4-Y	RPS4-X
Human	Housekeeping Ubiquitous Turner Gene	Housekeeping Ubiquitous No X-inactivation
Mouse	No RPS4-Y Evolved out of existence	Housekeeping Is X-inactivated
	ZFY	ZFX
Human	Housekeeping Ubiquitous Turner Gene	Housekeeping Ubiquitous No X-inactivation
Mouse	Male specific function <i>only</i> TWO COPIES ONLY	Housekeeping UBIQUITOUS Is X-inactivated

There were ten cases of translocations inherited from the infertile male (.92%), and these children are also likely to be infertile. Nine of these ten were balanced translocations in normal newborns. The one (0.09%) unbalanced translocation, was diagnosed at amniocentesis and was terminated. Since approximately 2% of oligospermic infertile males have chromosomal translocations (compared to a controlled population of 0.25%), it is not surprising that 0.9% of ICSI offspring would inherit such a translocation from their father (Van Assche et al., 1996). Thus, on purely conventional cytogenetic evidence, approximately 2% of ICSI offspring might be expected to share their father's infertility.

The remarkable five-fold increase in de novo balanced translocations among ICSI offspring (0.36% compared to 0.07%) is of great concern. Only 20% of balanced translocations are de novo, and 80% are inherited (Jaoobs et al., 1992). De novo balanced translocations are usually of paternal origin (84.4%) and obviously most of the inherited balanced translocations in ICSI patients would come from the father (Egozcue et al., 2000; Olson and Magenis, 1988). Balanced translocations which are associated with male infertility thus originally arose de novo in the testis of an otherwise fertile father, or his paternal ancestors, in 0.07% of a control population. Much more

frequently, de novo balanced translocations (albeit still a low percentage of only 0.36%) arise in the testis of infertile men undergoing ICSI and are transmitted to their offspring. The deficient testis appears not only to be at risk of transmitting inherited autosomal cytogenetic defects, but also of producing a greater number of de novo cytogenetic defects.

TABLE 5.8 KARYOTYPE ANOMALIES IN 1,082 PRENATAL DIAGNOSIS

Abnormal Karyotypes On 1,082 Prenatal Tests	Maternal Age (years)	Number	Percent	Percent in Literature
De novo chromosomal aberrations		18	1.66	0.445
Sex-chromosomal:		9	0.83	0.19, 0.23
45, X	37			
46, XX/47, XXX	44			
47, XXX (2 children)	32, 37			
47, XXY (4 children)	26, 28, 28, 32			
47, XYY	25			
Autosomal:		9f	0.83	0.21, 0.61
Trisomy 21 (5 children)	32, 33, 37, 41, 41	5	0.46	0.14
structural		4	0.36	0.07
46, XXY, t (4;5)	30x			
46, XX, t (2;15)	30			
46, XX, t (2;13)	36			
46, XX, inv (1qh)	39			
Inherited aberrations		10	0.92	0.47
balanced		9	0.83	0.45
unbalanced		1	0.09	0.023
Total aberrations de novo + inherited		28	2.5	0.92, 0.84

(Bonduelle et al., 1995; Bonduelle et al., 1996; Bonduelle et al., 1998; Bonduelle et al., 1999)

The incidence of congenital abnormality in ICSI children (2.3%) is no greater than in every normal population studied (Bonduelle et al., 1995, 1996, 1998a, 1998b, 1999). Even the few reported ICSI offspring of Klinefelter's patients have been chromosomally normal (Palermo et al., 1998; Tournaye et al., 1996; Staessen et al., 1996; Levron et al., 2000). There is no greater incidence of autosomal aneuploidy than what is predictable from maternal age. Sex chromosome aneuploidy (0.83%) in ICSI offspring is not an unacceptably high incidence, although it is clearly greater than normal (0.19%). Thus, the evidence based on cytogenetic and pediatric follow-up of ICSI offspring is very reassuring, despite the probable occurrence of infertility and sex chromosomal disorders in a small percentage of cases. Study of the Y

chromosome, however, leads to even greater concern regarding the future fertility of these children.

Y DELETION STUDIES OF ICSI OFFSPRING

Microdeletions on the long arm of the Y chromosome do not appear to adversely effect the fertilization or pregnancy results either in severely oligospermic men, or in azoospermic men from whom sperm were successfully retrieved (Silber et al., 1998). There have been concerns registered that the ICSI results might be poorer with Y-deleted men, but in larger series, that has not been the experience (Van Golde et al., 2001; Silber, 2001) (Table 5.3).

Thus far, all of our male offspring from Y-deleted men have had the same Y deletion as their infertile father (Page et al., 1999). Fathers, brothers, and paternal uncles of the infertile men, were also examined for Y deletions and fertility. Y deletions in our infertile males were *de novo* for the most part. That is, the fertile fathers of the infertile Y-deleted patients had no Y deletion. However, all male offspring from ICSI procedures involving these Y-deleted men had their father's Y deletion transmitted to them without amplification or change (Fig. 5.11).

The idea that the Y deletion would be transmitted to the son is not as obvious as it might at first seem. If a few foci of spermatogenesis in the testis of a severely oligospermic or azoospermic Y-deleted man were present because of testicular mosaicism, it would seem very possible that the few areas of normal spermatogenesis within such a deficient testis of a Y-deleted man might actually have a normal Y chromosome. In that event, one could have expected the sons of these patients undergoing ICSI not to be Y-deleted. For example, thus far all the sons of Klinefelter's patients have been normal 46, XY (Palermo et al., 1998; Tournaye et al., 1996; Staessen et al., 1996; Levron et al., 2000). Thus, it is not at all obvious, intuitively, that this Y deletion had to be transmitted to the son. However, increasing experience seems to indicate that the Y deletion of the sterile father is, in fact, transmitted to the son, and we no longer have to just speculate about it.

It remains to be determined whether non Y-deleted fertile or infertile men have mosaic deletions in their testis. If so, then *de novo* Y deletions would also be found more frequently in the brothers of our Y-deleted patients, or in ICSI offspring of infertile men (even those who have no Y deletion) than would otherwise be expected to occur in a normal newborn population (Kent-First et al., 1996). However, what we now know from the detailed sequence studies of the AZFa and AZFc regions of the Y chromosome gives us a much better picture of how Y deletions commonly occur, and how they are transmitted to offspring.

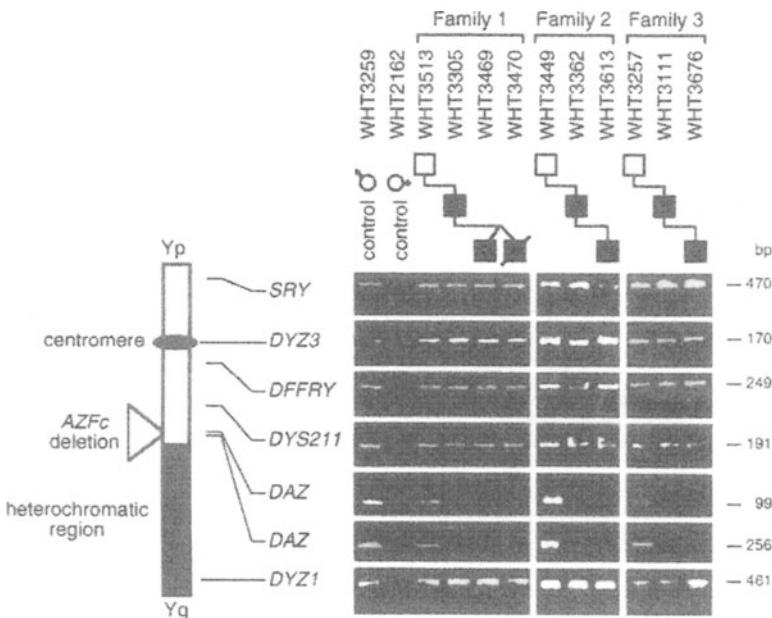


FIGURE 5.11. The AZFc Y deletion present in the azoospermic or severely oligospermic father is not present in his father, but is transmitted to his sons via ICSI. (Page, Silber, Brown, 1999).

MECHANISM OF DE NOVO APPEARANCE OF Y DELETION, AND ITS TRANSMISSION TO FUTURE GENERATIONS

The first region of the Y chromosome that was completely sequenced was AZFa because it was a region of the Y with very little repetitive sequences, and relatively amenable to study. Now, the more daunting AZFc region (with large areas of sequence identity) has also been very recently sequenced (Kuroda-Kawaguchi et al., 2001). The sequence of AZFa, revealed it to span approximately 800,000 nucleotide bases (800 KB), and was bounded on each side by a proximal breakpoint area and a distal breakpoint of around 10,000 bases (10 KB) of 94% sequence identity with each other. Furthermore, the sites of these breakpoints (even with conventional mapping) in most infertile men with AZFa deletions were indistinguishable from each other. Within these 10 KB breakpoint regions, the site of AZFa deletion almost uniformly fell within smaller domains (447 BP to 1285 BP) of these 10,000KB breakpoints that exhibited *absolute sequence* identity (Sun et al., 2000). Indeed, the sequencing of deletion junctions of most AZFa-deleted patients revealed that homologous ("illegitimate") recombination had occurred between identical areas of proviruses that bounded each side of this 800,000 base region, allowing the entire intervening segment to drop out. The repeat

areas of absolute sequence identity proximal and distal to a common area of deletion gives us a clue to the mechanisms of these Y deletions. With AZFa, the sequence repeats are caused by an ancient intrusion of a retrovirus into that region of the human Y. For AZFc, the situation is similar but occurs for a different reason on a vast scale of unprecedented and much more massive lengths of repeats.

The findings in AZFa give a clue to what is operating in the more common areas of deletion, such as AZFc. The sequenc of AZFc reveals the same mechanism as for AZFa but on a grander scale. Large domains of absolute sequence identity become easy sites for drop-out of large chunks of DNA, as the boundaries of absolute sequence identity illegitimately recombine with each other (Tilford et al., 2001; Kuroda-Kawaguchi et al., 2001). Because of amplification and inversions in the most ancient areas of divergence of the non-recombining Y, the whole situation is a set-up for deletion and degeneration. The very repetitive nature of the Y chromosome, that made sequencing and finding small deletions or point mutations so difficult, is the cause of its instability both over an evolutionary time frame, as well as in our current infertile patient population. Y deletions large enough to be detected with our outmoded maps occur in about 1/2000 births because of these vast areas of absolute sequence identity.

The AZFc region of the Y, which is the most common deletion site, spans 3.5 megabases of DNA, and is thus hardly a “microdeletion” (as it is often called). It is composed of three giant palindromes constructed from six families of cmplicons (i.e., long areas of absolute sequence identity). It contains 19 transcriptional units composed of seven different gene families, only one of which is DAZ. The 3.5 mB AZFc region is bounded on each side by 229,000 long areas of near absolute sequence identity (99.9%). Unlike AZFa, the “breakpoints” of sequence identity did not come from an ancient retrovirus, but rather from the very nature of the evolution of the Y chromosome because of the failure of recombination. There are multiple other areas on the Y (and in AZFc) that have either direct or inverted sequence repeats. Direct repeat sequences will result in common deletions due to homologous recombination. Inverted repeats will result in “isodicentric” translocations also because of homologous recombination. Thus, we can expect to find many other, smaller deletions that have previously escaped detection by crude, non-sequence based STS mapping.

It may very well be that smaller deletions, taking out less genes, or point mutations in just one or two copies of identical genes that occur in multiple copies, could account for many more cases of male infertility, or perhaps more moderate degrees of spermatogenic failure (e.g. $> 2 \times 10^6$ to 20×10^6 sperm/cc). The large “micro-deletions” thus far reported in the literature are for the most part de novo, but certainly some men with severe oligospermia can naturally father children (about 5%). Men with more moderate degrees of oligospermia may father children even more easily, and thus smaller Y

deletions causing male infertility may indeed not as often be de novo. In any event, as more genetic causes of spermatogenic failure (severe or mild) come to light, there will be an increased awareness of the possible transmission of this infertility to future generations.

THE X CHROMOSOME AND MALE INFERTILITY

It has been theorized that the Y is not the only sex chromosome that accumulates genes which benefit spermatogenesis over an evolutionary time span (Rice 1984, 1992; Brooks 2000; Fisher 1931). As the X chromosome (240 to 320 million years ago) differentiated from the Y, the sexually antagonistic gene theory favors the emergence of genes on the X also that benefit the heterogametic sex (with mammals of course, that is the XY male) and are detrimental to the homogametic sex (the XX female) *if these genes are recessive*. For example, a rare recessive evolutionary mutation on the X that favors spermatogenesis would be preferentially passed on to male offspring who by virtue of a higher sperm count would then continue to pass down this favorable X mutation to his offspring. Such a recessive mutation (favorable to spermatogenesis) in an autosome would be lost to future generations. Thus, we can also anticipate an accumulation on the X chromosome (as well as the Y) of male benefit recessive genes.

In fact, RT-PCR subtraction studies of spermatogonia in mice have demonstrated that a large fraction of genes which are expressed exclusively in pre-meiotic male germ cells, are indeed X chromosomal in origin (Wang et al., 2001). Eleven of the 36 genes that were expressed specifically in mouse spermatogonia were found exclusively on the X chromosome. Since the X chromosome is so well conserved in all mammals (as explained earlier by the universal development of X inactivation in mammalian evolution), it seems very likely that evolution has also conferred on the human X chromosome a large portion of the burden for spermatogenesis. Thus, a search for detrimental mutations on the human X in infertile males is also likely to be very rewarding. Thus, the failure to identify a Y deletion gives no assurance whatsoever that a genetic cause for infertility won't be transmitted to the ICSI offspring either via the X, or even autosomes.

CONCLUSIONS

The presence of Y deletions does not decrease the fertilization or pregnancy rate for azoospermic and severely oligospermic ($<2 \times 10^6$) men. Thus far the sex ratio of delivered children is apparently equal and the children are karyotypically normal. However, the Y deletion (and presumably infertility) is transmitted to the male offspring (Page et al., 1999). Although using standard STS mapping, Y deletions occur in only 10% of azoospermic

and severely oligospermic men, sequenced based mapping (now just available) may increase that percentage significantly.

There are most likely many spermatogenesis genes involved in male infertility, and we have barely scratched the surface with what have been, up till now, very crude mapping techniques on the Y chromosome. Whether or not these gross "microdeletions" currently reported in the literature are found in an infertile male patient, does not obviate the likelihood of there being a genetic cause for his azoospermia or severe oligospermia. If a defective gene (or genes) is located on his Y chromosome, then his male offspring will most likely inherit his problem. However, there are also many genes on the X chromosome, and throughout the genome, that impinge upon spermatogenesis that are not thus far identified by our currently crude mapping procedures. The recognized failure of any conventional therapy to improve spermatogenesis infers a genetic origin for most spermatogenic defects (Silber 2000a; Silber 2000b; Silber 2001). These numerous genes may also be responsible for many cases of male infertility. Therefore, sons, and even daughters, may inherit the defect or be carriers.

It is clear that a negative Y microdeletion assay by currently popular methods does not rule out genetic abnormality. Therefore, in our view, genetic counseling should be provided to all infertile males, whether or not an abnormality is detected and whether or not Y deletion assays have even bothered to be performed. Although karyotyping certainly should be routinely performed for infertility patients (because of the risk of miscarriage and abnormal offspring resulting from either sex chromosome abnormalities, or unbalanced translocations), Y deletion testing may not be mandatory yet, because it is still very crude, and negative results should not be at all reassuring. Furthermore, some "deletions" may only be polymorphisms, and not of clinical significance. It is apparent that there is likely to be frequent transmission of male infertility from the ICSI father to his male (or even female) offspring regardless of current testing. Every couple must decide for themselves whether they wish to consider this risk. In our experience, most such couples, even when well informed, choose to have ICSI despite this risk. Thus, continued long-term clinical and molecular study of ICSI offspring is mandatory.

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CHAPTER SIX

SPERM ANALYSIS AND PREPARATION UPDATE

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INTRODUCTION

Since the introduction of Intra Cytoplasmic Sperm Injection (ICSI) in 1992 in Assisted Reproduction Techniques (ART), the importance of sperm analysis and sperm preparation techniques has become debatable. Indeed: with this technique only few spermatozoa are necessary to obtain a successful fertilization, embryo development and a healthy pregnancy. The success of ICSI notwithstanding, it should be understood that ART includes other fertility treatments such as intrauterine insemination, and even with in vitro fertilization (IVF), most attempts involve conventional insemination. The relevance of semen analysis ART remains essential both in the determination the mode of infertility treatment and in the evaluation of failed cycles where male factor parameters become critical in the decision of the next step. This chapter discusses the elements involved in the study and preparation of a human sperm for ART, with the aim of identifying normal spermatozoa that if necessary, can be used in the isolation of a single healthy spermatozoon for ICSI.

EJACULATED SPERM SAMPLE

The analysis of the semen sample provides important information about the quality of the sperm. Parameters like concentration, motility and morphology contribute to defining the ‘normality’ of a semen sample. The *normal semen parameters* established by the World Health Organization (1999) are shown in Table 6.1. However, an increasing number of clinical studies have questioned the WHO threshold values for classification of *subfertile or fertile* patients. Studying parameters such as concentration, morphology and motility in a large group of patients in prospective

revised. (Ombelet et al., 1997a, 1997b; Gunalp et al., 2001; Guzick et al., 2001). The proposed threshold values for *sub fertile* patients are sown in Table 6.2.

SPERM ANALYSIS

The evaluation of the sperm sample should be standardized beginning at the moment of collection to the final counting of normal spermatozoa in the fixed sample. A continuous quality control of this analysis is therefore of utmost importance. Different countries have established an objective quality control system. Often they are under the control of the Ministry of Health (Franken et al., 2000a; 2000b; WHO laboratory manual, 1999 and ESHRE monographs, 2002). Practical details on all laboratory techniques can be found in the following reference papers: Dale and Elder, 1997; WHO laboratory manual, 1999 and ESHRE monographs, 2002.

TABLE 6.1 NORMAL SEMEN PARAMETERS

Volume	$\geq 2.0 \text{ ml}$
pH	≥ 7.2
Sperm concentration	$\geq 20 \text{ million spermatozoa/ml}$
Total sperm number	$\geq 40 \text{ million spermatozoa/ml}$
Motility	$\geq 50\% \text{ motile (grade a+b) or } \geq 25\% \text{ grade a}$ within 60 minutes of ejaculation
Morphology	$\geq 15\%$
Vitality	$\geq 75\% \text{ excluding dye}$
White blood cells	$< 1 \text{ million cells/ml}$
MAR test	$< 50\% \text{ motile spermatozoa with beads bound}$
Immunobead test	$< 50\% \text{ motile spermatozoa with adherent particles}$

SEmen COLLECTION

The semen sample should be collected at the laboratory in a special sterile container after a 2-3 day period of abstinence. Alternatively, the sample may be brought in from home within 1 hour of ejaculation and should be maintained at body temperature during transit. No lubrication should be used while obtaining the sample as it may be toxic to sperm. The sample may have to be obtained during intercourse, and a special condom designed for this purpose should be provided. A minimum of two semen. A minimum of two semen analyses have to be performed several weeks apart because sperm counts can fluctuate.

TABLE 6.2 THRESHOLD VALUES FOR SUBFERTILE MEN

	Ombelet et al., 1997	Guzick et al., 2001	Gunalp et al., 2001
Sperm concentration	$14.3 \cdot 10^6/\text{ml}$	$13.5 \cdot 10^6/\text{ml}$	$9.10 \cdot 10^6/\text{ml}$
Motility – grade a	28%	32%	14%
Morphology Strict criteria	5%	9%	5%

MACROSCOPIC EVALUATION OF SEMEN

During the initial macroscopic investigation of the sample, volume, colour pH, viscosity and concentration should be recorded as shown in Table 6.3.

TABLE 6.3 MACROSCOPIC CHARACTERISTICS OF A SEMEN SAMPLE

Volume	2 ml	Normal
	< 2ml	Possible retrograde ejaculate or prostate deficiency
	>> 2ml	Long period of abstinence
Color	Grey Opalescent/clear/ Gelatinous bodies	Normal
	Yellow	Long period of abstinence Intake of B-vitamins
	Reddish-brown	Erythrocytes
Viscosity after liquefaction	High	Interferes with sperm motility Lack of homogeneity of the sample
pH	≥ 7.2	Normal
	< 7	Azoospermia Obstruction of ejaculatory ducts Bilateral congenital absence of vas deferens

MICROSCOPIC EVALUATION

The first microscopic evaluation of the sample includes assessment of motility and agglutination of spermatozoa and the presence of cellular

elements other than spermatozoa, which would include red and white blood cells, or epithelial cells. The occurrence of bacteria must also be noted.

SPERM CONCENTRATION

Correct sperm concentration should only be determined using a hemocytometer as other types of counting chambers give incorrect results (ESHRE monographs, 2002). Samples in which no spermatozoa are readily detected should be centrifuged to determine whether they exist in pellet, and centrifugation at > 3000g for 15 minutes is recommended. Only when no spermatozoa are found after a complete and systematic search in the pellet should the sample be classified as azoospermic. The different classifications of sperm samples according to count, motility and morphology are shown in Table 6.4.

TABLE 6.4 NOMENCLATURE ACCORDING TO WHO LABORATORY MANUAL, 1999

Normozoospermic	Normal ejaculate
Oligozoospermia	Sperm concentration less than the reference value
Asthenozoospermia	Motility less than the reference value
Teratozoospermia	Normal forms less than the reference value*
Azoospermia	No spermatozoa present

- Not defined in WHO Gold Book

ASSESSMENT OF MORPHOLOGY

At least two smears should be made from the fresh semen sample for duplicate assessment and to address problems with staining. Papanicolaou stain is the method most widely used in andrology laboratories and in our experience, provides good staining of the spermatozoa and other cells. It permits staining of the acrosomal and post-acrosomal regions of the head, cytoplasmic droplets, the midpiece, and the tail.

ANALYSIS

At least 2x 200 spermatozoa should be evaluated per sperm sample. Strict criteria should be applied when assessing the morphological normality of the

spermatozoon (Menkveld et al., 1990, Ludwig, 1987). The sperm head, neck and midpiece and tail are closely evaluated. A normal spermatozoon has an oval shaped head with a length of 4.0 to 5.5 μm and a width 2.5 - 3.5 μm . The length-to-width ratio should be 1.50 to 1.75. These ranges are the 95% confidence limits for Papanicolaou-stained sperm heads. The midpiece should measure less than 1 μm in width, about one and a half times the length of the head, and be attached axially to the head. Cytoplasmic droplets should be less than half the size of the normal head. The tail should be straight, uniform, thinner than the midpiece, uncoiled and approximately 45 μm long. This classification scheme requires that all 'borderline' forms be considered abnormal (Kruger et al., 1986; Menkveld et al, 1990), hence the term 'strict' criteria. With these criteria, the predictive value of sperm morphology for fertilization *in vitro* has been demonstrated in several important studies (Kruger et al, 1986, 1988; Ombelet et al., 1995). Table 6.5 summarises the morphological characteristics examined in a typical sperm analysis.

Occasionally, many of the spermatozoa have a specific structural defect such as failure of the acrosome to develop, causing ta 'small round-head defect' termed 'globozoospermia'. Failure of the basal plate to attach to the nucleus at the pole opposite the acrosome causes heads and tails to detach on spermiation. The heads are absorbed and only tails are found in the semen giving these structures a defect termed 'pinhead'. Pinheads) are not counted as head defects because they only rarely possess chromatin or other head structures anterior to the basal plate. If many pinheads are seen this should also be noted on the formal report of the semen analysis.

Transmisison electron microscopic analysis is advised when a sperm sample demonstrates total asthenozoospermia. This additional step is taken in these selected cases in order to define if the origin of immotility such as immotile cilia syndrome.

MOTILITY

A simple grading system is recommended to provide a relatively effective assessment of motility without the need for complex equipment such as computer based systems that use image analysis software (computer aided sperm analysis (CASA), which are not be discussed. At least five microscopic fields are assessed systematically to classify a minimum of 200 spermatozoa. Hyperactive spermatozoa are not included in the WHO standard protocol shown in Table 6.7 but give a qualitative information after preparation. These spermatozoa have a very rapid progressive motility ($>50\mu\text{m/s}$).

**TABLE 6.5 SUMMARY OF MORPHOLOGICAL DEFECTS
(WHO LABORATORY MANUAL, 1999)**

Head defects	<ul style="list-style-type: none"> -large small, tapered, pyriform-round -amorphous heads -vacuolated heads (> 20% of the head area occupied by unstained vacuolar areas) -small acrosomal area (< 40% of the head area) -large acrosomal area (>70% of the head area) -double heads -any combination of these
Neck and midpiece defects	<ul style="list-style-type: none"> -‘bent’ neck: the neck and tail form an angle of greater than 90° to the long axis of the head -asymmetrical insertion of the midpiece into the head -thick or irregular midpiece -abnormally thin midpiece -any combination of these.
Tail defects	<ul style="list-style-type: none"> -short -multiple tails -hairpin -broken tails -bent tails (> 90°) -tails of irregular width, coiled tails -any combination of these
Cytoplasmic droplets	greater than one-third of the area of a normal sperm head. The droplets are usually located on the side of midpiece.

TABLE 6.6 MOTILITY GRADING ACCORDING TO THE WHO STANDARDS (1999)

Grade A	Rapid progressive	$\geq 25\text{mm/s}$ 1 monitor square or 5 sperm heads lengths
Grade B	Slow progressive	5-24 mm/s
Grade C	Non-progressive	<5 mm/s
Grade D	Immotile	

DO WE NEED TO PREPARE SPERM SAMPLES?

The ejaculate is a composition of spermatozoa of different qualities suspended in secretions of the epididymis, the testis, prostate, seminal vesicles and the bulbourethral glands. Cells other than spermatozoa are also present in the ejaculate. These include epithelial cells from the urinary tract, prostate cells, spermatogenic cells and leukocytes. Reactive oxygen species (ROS) are also present in the ejaculate. They can either be produced by the different germ cells or by leukocytes and can be detrimental for the fertilizing potential of the spermatozoa owing to their oxidation of lipids in the plasma membrane (Gil-Guzman et al., 2001). Several studies indicate that the preparation, isolation and concentration of morphological normal and motile spermatozoa are essential to and necessary for any ART technique (capacitation test, IUI, IVF ICSI or cryopreservation), and two preparation techniques have been shown to be clearly superior: the density gradient centrifugation and the glass-wool filtration techniques. They clearly improve the number of morphological normal spermatozoa with grade 'A' motility and with normal chromatin condensation in the prepared sample (Berlau J et al., 2000; Tomlinson et al., 2001a, 2001b; Sakkas et al., 2000; Hammadeh et al., 2001; Erel et al., 2000). Moreover the reduction of ROS and leukocyte concentration is significantly improved with the latter technique. The spermatozoa obtained from these preparations show reduced levels of chromatin and nuclear DNA anomalies and enhanced rates of nuclear maturity. An improved acrosome reaction as well as higher hypo-osmotic swelling test reaction (HOS) (Ding et al., 2000) for men with abnormal semen analyses are obtained after these modes of preparation when compared to traditional wash, swim-up and albumin gradient techniques.

SPERM PREPARATION TECHNIQUE: GRADIENT CENTRIFUGATION

The gradient may be composed of colloidal silica particles stabilised with covalently bound hydrophilic silane colloidal particles. The solution contains EDTA and glucose (Figure 6.1). The gradient is formed stepwise with layers of 90%, 70% and 40% solutions in order to select for specific cell diameters, and the first layer will retain epithelial and blood cells. The 70% layer will entrap immature sperm (round spermatids) and spermatozoa with abnormally shaped heads. In practice however, a dual gradient composed of 40 and 90% solutions is equally effective in isolating normal spermatozoa.

BIOCHEMICAL MARKERS

Determination of markers of sperm function, accessory sex gland secretion and the occurrence of silent male genital tract inflammation (asymptomatic) is of considerable diagnostic value in the evaluation of male infertility. The introduction of biochemical tests of male factor infertility has the advantage of providing standardized assays with a coefficient of variation characteristic of clinical chemistry, in contrast to biological test systems which can be inherently quite variable. Biochemical parameters may be used in clinical practice to evaluate the sperm fertilizing capacity (acrosin, aniline blue, ROS levels), to characterize male accessory sex gland secretions (fructose, alpha-glucosidase, and a specific prostatic markers such as PSA), and to identify men with silent genital tract inflammation (granulocyte elastase, C'3 complement component, coeruloplasmin, IgA, IgG, ROS) (Schill and Henkel, 1999; WHO manual, 1999).

WHAT TO DO WITH THE SPERM ANALYSIS: TIMED INTERCOURSE, IUI, IVF OR ICSI?

It is obvious that the decision concerning which method of assisted reproduction to use in ART is influenced by the semen result and other male and female factors such as the presence of ovulatory defects, endometriosis, tubal factors, endocrine dysfunction or abnormalities, antisperm antibodies, etc. In women whose fertility evaluation is normal, the following recommendations can be made, with the caveat that prospective randomized studies are largely absent lacking in this particular area of human reproductive medicine. If a properly timed postcoital test is normal (more than 5 spermatozoa with good progression per high powered field, HPF, 400 x), it may be appropriate to advise timed intercourse for at least three cycles with or without ovarian stimulation. Because conception is the only endpoint in these cases, ovarian stimulation with gonadotrophins is option of choice. This,

however, requires careful monitoring as the risks for multiple pregnancy and the ovarian hyperstimulation syndrome (OHSS) increase significantly with gonadotrophin stimulation. Therefore, timed intercourse in a natural cycle is preferable if the patient has a regular ovulatory cycle. With clomiphene citrate (CC, 50 mg for five days) for ovarian stimulation, the pregnancy rate per cycle will increase with an acceptable twin-rate of approximately 6 %. Before starting treatment, however, it is always necessary to discuss the advantages and disadvantages of ovarian stimulation with the couple.

The use of CC can be supported for three or more cycles depending upon such factors as the duration of subfertility and maternal. If treatment is unsuccessful, artificial insemination is the method of choice, if sperm quality is appropriate (>300,000 motile spermatozoa after preparation: Ombelet et al., 1995a). Artificial insemination can involve intravaginal (IVI), intracervical (ICI) or pericervical methods using a cervical cap. Other methods that are also in common use include intrauterine (IUI), intratubal (ITI) or direct intraperitoneal (IPI) insemination. IUI is the method of choice in most infertility programs, but for male factor cases, IUI and IPI result in comparable results (Evans et al., 1991; Gregoriou et al., 1993). However, IPI is a more invasive technique and should be specifically reserved for those cases in which IUI is difficult or impossible due to a non patent cervix.

According to published clinical outcome studies, as well as cost-benefit analysis, IUI following clomiphene citrate ovarian stimulation is probably the most effective treatment for moderate male subfertility (Goverde et al., 2000; Guzick et al., 1999). Most studies indicate that after preparation, 5 million motile spermatozoa (also termed inseminating motile count, or IMC) are required for IUI to be effective. In our experience, both IMC and morphology are important predictive factors.

In a selected group of patients with no demonstrable CC-resistance and a normal ovarian response following CC-stimulation (max. 3 follicles with a diameter of > 16 mm at the time of human chorionic gonadotropin, hCG, administration), we have found that IUI combined with CC-hCG is a safe and cost-effective first-line treatment when an IMC of more than 1 million spermatozoa are available for insemination, with a cumulative baby-take-home rate of 25 % after three cycles. With an IMC of < 1 million, CC-IUI remains important as a first-choice therapy provided the morphology score is more than 4% (cumulative pregnancy rate of 22 %; (Ombelet et al., 1997c).

Many studies report a significant decline in cycle fecundity after the third or fourth cycle of IUI (Comhaire et al., 1994; Dodson and Haney, 1991; Nan et al., 1994; Plosker et al., 1994). These couples do not seem to benefit from this method of treatment and should be advised to consider other treatment options such as IVF or IVF-ICSI. In our view, IUI can be used as a very important first-line treatment in most cases of moderate and severe male subfertility before starting IVF or ICSI, providing that at least one million motile spermatozoa can be recovered.

Concentration of layer	100% PureSperm	EBSS suppl
90%	9 ml	1 ml
70%	7 ml	3 ml
40%	4 ml	6 ml

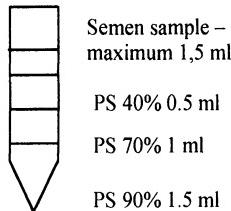


FIGURE 6.1. This figure shows the design of a typical gradient centrifugation system for sperm preparation using a stock solution of 100 % PureSperm (Nidacon, Goteborg, Sweden). The density gradient is centrifuged for 20 minutes at 2000g and the 90 % layer is transferred to a fresh tube containing 10ml of sperm wash medium. The suspension is washed by centrifugation for at 2000g 10 minutes. The supernatant is discarded and the washing procedure repeated. The pellet is resuspended in 1 ml of culture medium and the concentration and motility verified microscopically. The sample is equilibrated for at least 20 minutes in the incubator to achieve the appropriate pH and temperature. When sperm is intended for IVF, up to 100,000 grade A are used for insemination when morphology is normal (>10 % normal forms), usually in a 1 ml volume of culture mediumt. With poor morphology (< 10 % normal forms) up to 500,000 good motile sperm (grade A) are used.

TESTICULAR SPERMATOZOA

INTRACYTOPLASMIC SPERM INJECTION WITH TESTICULAR SPERM

Schoysman et al (1993) described the ability of testicular spermatozoa to fertilize a human oocyte after ICSI. This study was the first to report a pregnancy after embryo transfer with spermatozoa obtained from a testicular biopsy in a patient where epididymal sperm retrieval failed. Patients suffering from obstructive or non-obstructive azoospermia were offered a new treatment for their infertility. Many centers now offer testicular sperm preparation in combination with ICSI (Palermo et al., 1999).

THE ERA OF TESE OR TEFNA?

Different methods for the surgical retrieval of testicular tissue have been described. Testicular sperm extraction (TESE) is usually involves an open

biopsy that is performed under local or general anaesthesia as follows: a small incision is made in the tunica albuginae and the small portion of extruding testicular tissue is removed for sperm (Schoysman et al., 1993). If no spermatozoa are found, the biopsy is repeated in another area of the testis, or even in the second testis until a quantity of spermatozoa sufficient for ICSI are present. For men with active spermatogenesis, with or without obstruction, or in situations where no spermatozoa are retrieved after epididymal sperm puncture, a 'blind' testicular fine needle aspiration (TEFNA, or TESA, testicular sperm aspiration; Craft et al., 1995) may be performed. In this case, a 23 gauge needle or biopsy gun needle is introduced in the testis and by means of negative suction, tubular material is aspirated into culture medium. An immediate microscopic inspection determines whether spermatozoa are present and whether additional areas need to be sampled. This technique yields a much lower number of spermatozoa that can be used for ICSI or cryopreservation when compared to recovery rates associated with an open biopsy in similar cases. Originally, it was thought that this technique would be less invasive for the testis than the open biopsy (Schlegel and Su, 1997). However, Shufaro et al. (2002) reported findings from a rat model demonstrating that TEFNA inflicted severe, progressive and irreversible damage, as well as atrophy in the architecture of the semineferous tubules that were directly in the pathway of the biopsy needle. In contrast, TESE caused only localized scarring and fibrosis such that most of the remaining testicle was intact and unaffected. Combining both parameters (yield of sperm recovery and invasiveness of the collection technique), the open biopsy procedure is clearly the method of choice.

DO WE NEED TO PREPARE TESTICULAR SAMPLES?

In addition to mature spermatozoa, testicular samples often contain debris and unwanted cells such as immature spermatozoa (round spermatids, elongating and elongated spermatozoa,), spermatocytes and spermatogonia, as well as somatic contaminants including Sertoli cell nuclei, red blood cells, remnants of the seminiferous tubules, cytoplasm of round spermatids and intact Sertoli and Leydig. Preparation of testicular samples is more complex than used for epididymal spermatozoa. In order to express spermatozoa from the seminiferous tubules that can be used for ICSI, the standard method is to shred the biopsy sample (whether a large open biopsy piece or a small needle biopsy sample) by repeatedly pulling the sample apart with tuberculin needles or glass slides. The sample is then compressed between glass slides, vortexed and examined for spermatozoa under high power (Kahraman et al., 1996). Modifications to the technique are maceration with microgrinders, enzymatic treatment or treatment of the sample with erythrocyte lysing buffer (Nagy et al., 1997). Baukloh (2002) reported no difference between these two methods with respect to rates of fertilization, embryo development and implantation.

Samples can also be expressed through a 40 μm pore cell strainer, which effectively removes cellular and tubular debris (Nijs et al., unpublished). Stewart (1998) found that unravelling individual tubules with a forceps and compressing the tubule fragments starting at the midsection and moving progressively to the ends was an effective method for expressing functional spermatozoa. Fresh testicular sperm samples can be further processed by simple washing in medium or by discontinuous gradient centrifugation to remove ancillary cells and debris. The latter procedure is preferred because it produces a neat suspension of testicular spermatozoa. In order to protect sperm from potential ROS damage, the concentration of human serum albumin, or whole serum, is usually doubled during the preparation of these sperm. Higher protein or serum concentrations also reduce the possibility of sperm sticking to the ICSI needle during insemination. Typically, while approximately half of the testicular sperm samples show no motility at the time of preparation, immotile spermatozoa can still be used for ICSI as they have the same fertilizing potential as motile spermatozoa that occur after approximately 4 hours of culture (Nijs et al., 1996). However, when the testicular sample is placed in culture, the proportion of motile sperm is usually highest (91%) after 24 hours of incubation (Nijs et al., 1997) making the identification of living spermatozoa relatively unambiguous. Therefore when flexibility is possible in the timing of the testis biopsy, they should be scheduled a day in advance of the ovum retrieval. ICSI with testicular spermatozoa is similar to ICSI with ejaculated spermatozoa. After the selection of a morphologically normal and preferably motile spermatozoa from the sperm droplet (see below), the sperm is immobilised in polyvinylpyrrolidone (PVP) and injected in the oocyte. Although chromatin condensation and nuclear maturation are incomplete in testicular spermatozoa, the outcome of fertilization with testicular spermatozoa is similar to that of ICSI with ejaculated spermatozoa (Hammadeh et al., 1999).

Although testicular sperm extraction (TESE) in combination with ICSI is the method of choice to treat male infertility due to obstructive or non-obstructive azoospermia, a determining factor in the success of ICSI with TESE is clearly related to sperm recovery. Overall, sperm recovery rates in non-obstructive azoospermic men varies between 40 and 70% (Friedeler et al., 1997; Tournaye et al., 1997; De Croo et al., 2000). Seo and Ko (2001) found a clear correlation between the testicular sperm recovery rate and testis histopathology. Compared to Sertoli cell-only syndrome, the odds of successful sperm retrieval were 44.3 times higher in cases of severe hypospermatogenesis and 8.4 times in men with maturation arrest. Whether spermatozoa can be recovered from azoospermic patients is related to the underlying etiology. When testicular histology is normal, as men with obstructive azoospermia or germ-cell hypoplasia, sperm recovery is virtually always successful. For men with germ cell aplasia or maturation arrest, the

yield of sperm extraction is unpredictable and variable (Tournaye et al., 1996).

With respect to outcome in men with non-obstructive and obstructive azoospermia, data concerning sperm quantity and quality is controversial. Several reports describe significantly reduced fertilization and pregnancy rates in cases of non-obstructive azoospermia (Fahmy et al., 1997; Mansour et al., 1997). . Ubaldi et al. (1999) reported significantly lower implantation rates after ICSI with testicular spermatozoa in non-obstructive azoospermia when compared to a matched control group with ejaculated spermatozoa, and to cohorts with obstructive azoospermia but normal spermatogenesis. In the study reported by Ghazzawi et al. (1998), the live birth rate per embryo transfer was significantly reduced in patients with non-obstructive azoospermia (10.0%) compared to the cohorts of men in which ejaculated sperm (21.0%) or epididymal (22.0%) sperm were used for ICSI. This is in contrast to findings described in other reports (Kahraman et al. (1996; Palermo et al, 1999; De Croo et al., 2000) which described lower fertilization rates in cases of non-obstructive azoospermia. Despite reduced fertilization, rates in these instances, pregnancy rates were unaffected. In contrast, Bukulmez et al. (2001) found no significant difference in outcome in cases of obstructive or non-obstructive azoopermia.

Lewin et al.(1999) demonstrated a clear correlation between the number of spermatozoa recovered and fertilization in cases of non-obstructive azoospermia. The fertilization rate may also be effected by different types of spermatogenetic defects present in the non-obstructive group. In cases of severe spermatogenetic defects, such as Sertoli Cell Only syndrome, severe hypospermatogenesis or maturation arrest can be associated with the occurrence of sperm that have not completed cytoplasmic maturation. In the study by De Croo et al. (2000), fertilization rates with these sperm were significantly lower than in cases of germ cell hypoplasia or Sertoli Cell Only syndrome. Tournaye et al. (1996) reported lower Fertilization rates in patients with maturation arrest (45.7%) and Sertoli Cell Only syndrome (44.0%) when compared to instances of germ cell hypoplasia (67.8%). Since maturation arrest and germ cell aplasia are assumed to have a genetic origin, genetic factors might also explain the decrease in fertilization rate (Vot et al., 1992). Palermo et al. (2002) analysed the genetic normality of testicular spermatozoa and found a higher incidence of chromosomal abnormalities, of which sex chromosome aneuploidy was the most predominant, in sperm from men with non-obstructive azoospermia. This finding illustrates the importance of considering potential genetic deficiencies in the counselling of patients who require ISCI for these types of male factor infertility (see Silber, this volume).

DIAGNOSTIC OR PARALLEL BIOPSY?

One way to minimise the number of surgical procedures on the testis is to cryopreserve biopsied testicular tissue and spermatozoa not used in the fresh ICSI-cycle. If a pregnancy is not achieved after ICSI with fresh testicular spermatozoa, thawed spermatozoa can be used in subsequent cycles. Even in cases of a diagnostic testicular biopsy , cryopreservation of testicular spermatozoa avoids a second biopsy, which is of particular benefit in men with in patients with non-obstructive azoospermia and small testes. Therefore, preference should be given to diagnostic testicular biopsies that are planned before the start of the ICSI-stimulation treatment. If the biopsy is negative, other options such as the use of donor sperm become evident.

CRYOPRESERVATION OF HUMAN SPERM

Cryopreservation of human spermatozoa is routine procedure in most IVF/ART programs. Human spermatozoa have a specific physical response to cryopreservation that requires the use of specialized protocols that avoid cryodamage, which can include impaired motility, reduced viability, or alterations at the molecular and cellular levels which can impair fertilizing potential. Because testicular spermatozoa are immature and recovered in small numbers, special techniques have been developed to enhance viability after thawing.

INDICATIONS FOR SPERM CRYOPRESERVATION

Spermatozoa or immature sperm cells obtained from an ejaculate, the epididymis or the testis can be cryopreserved in order to provide a sufficient quantity after thawing for different ART treatments (Figure 6.2). For IUI or conventional IVF, usually a sample containing a motile sperm concentration in excess of 5 million with > 4% normal forms is sufficient. When the motile sperm count is low (<5 million) and < 4% normal forms are present in the sample, ICSI is the method of choice (Nijs and Ombelet, 2000).

CRYOPHYSICS OF HUMAN SPERM CRYOPRESERVATION

In order for cells to survive cryopreservation, cryoprotective agents must be present during cooling and warming stages. Low molecular weight cryoprotective solutes protect cells by reducing the quantity of ice crystals formed within the cytoplasm. A controlled cooling rate permits the cytoplasmic water to exit the cell and freeze outside. This results in a gradual dehydration of the cytoplasm that tends not to compromise the retention of viability. For example, Gao et al. (1995) determined that human spermatozoa

could shrink to 0.75 times and swell to 1.1 times their iso-osmotic cell volume without losing their functional integrity.

Preservation of Fertility

Human Spermatozoa from ejaculate to be used in IUI/IVF/ICSI

- Prior to chemotherapy and/or radiotherapy or orchidectomy
- Prior to vasectomy

Testicular tissue to be used in ICSI after in vitro maturation or in vivo grafting prior to chemotherapy and/or radiotherapy for young boys

Donor sperm samples to be used in IUI or IVF

Conservation for infertility treatment in the future

Human Spermatozoa from ejaculate to be used in IUI/IVF

- Psychological reasons during IVF/ICSI treatment
- Physical absence during IUI/IVF/ICSI treatment
- Back-up cryopreservation

Epididymal or testicular spermatozoa, spermatids or testicular tissue

- Diagnostic epididymal or testicular sperm retrieval
- Surplus of epididymal or testicular spermatozoa during ICSI cycle

FIGURE 6 2. Indications for sperm cryopreservation

During this ‘osmotic processes.’ the concentration of cryoprotective agents within the cytoplasm greatly increases and if care is not taken during the cryopreservation process, these agents can reach toxic levels. An optimal cooling and warming rate determined for each cell type can avoid potential physical damage and toxicity. For thawing, cooling and warming rates are related to cell volume, surface area, water permeability, water permeating activation energy, and the type and concentration of cryoprotectants used (Watson, 1990). Cell permeating agents in common use such as glycerol will dehydrate by displacing water. Non-permeating agents such as sucrose are added to increase osmolarity in order to prevent damage by uncontrolled cellular swelling during the dehydration process. Proteins, amino acids and other permeating molecules may stabilize organelles, but are usually removed during the thawing process. and Certified cryopreservation solutions that are

commercially available offer a stable source of cryoprotective media. So-called 'in-house' prepared solutions, such as those containing egg yolk should be avoided. The successful cryopreservation of human spermatozoa is dependent upon optimal cooling and thawing rates, and most methods in current use while simple, are essentially crude. For example, samples are suspended in the vapor above liquid nitrogen resulting in differences in cooling rates between different cryocontainers (straws, plastic or glass ampoules). The optimal combination of a slow cooling process (<-100°C/min) with a rapid thaw procedure (400°C/min) provides a survival rate in the 90% range (Henry et al., 1993; Devireddy et al., 2000). Induction of ice nucleation, such as normally performed with embryo cryopreservation also improves the outcome with sperm (Morris et al., 1999). The traditional cooling in liquid nitrogen vapor should be avoided and replaced by an automated slow cryopreservation (-2°C/min to -7°C/ induction of ice nucleation/ -0.3°C/min to -30°C followed by plunge into liquid nitrogen: Stanic et al., 2000). However, specific cooling and thawing rates need to be determined for different cryoprotectant mixtures and cryocontainers (Critser et al., 1988; Hammitt et al., 1988).

CRYODAMAGE

Cell death due to intracellular ice formation and damage due to increased osmolarity of the cryoprotectant during cooling and thawing are the main problems associated with the cryopreservation of cells and for human spermatozoa, a significant reduction in viability can accompany thawing. Some studies have shown a cryopreservation-associated loss of 25-75% in motility in *normal* sperm samples, and higher losses if the original sample was characterized as oligoasthenoteratozoospermic (Hammadeh et al., 1999; Esteves et al., 2000; Nijs et al., 2000; O'Connell et al., 2002), and epididymal and testicular spermatozoa are even more susceptible to cryodamage than are ejaculated sperm (Nijs et al., 2000; Holden et al., 1997). The ability of spermatozoa to survive cryopreservation is patient-specific and assessments of membrane fluidity (anisotropy) appears to have predictive value with respect to outcome (Giraud et al., 2000). However, it is obvious that high rates of cryodamage will correspond to reduced fertilization rates in conventional IVF or IUI cycles (Critser et al., 1987; Crabbé et al., 1999, Hammadeh et al., 1999). For clinical ART, it is recommended that sperm samples always be prepared by swim up or gradient-centrifugation (Esteves et al., 2000).

CRYOPRESERVATION OF TESTICULAR SPERM

Methods for the cryostorage of human testicular spermatozoa and/or spermatids have been developed recently, but they differ from those used for

ejaculated spermatozoa. Since the former samples occur at low concentration, have very poor motility and are contaminated with sperm cells at different stages of maturation, the type and extent of cryodamage can significantly influence outcome. Successful cryopreservation of testicular material as intact biopsy specimens, isolated seminiferous tubules, or post preparation sperm should usually avoid repeat surgical biopsies for the affected patients(Crabbé et al., 2000; Stewart et al., 1998, Bahadader et al., 2000). Testicular sperm can be cryopreserved for several ICSI attempts, but they should first be isolated from testicular tissue/debris results (Allan et al., 1997; Crabbé et al., 2000; Ganiroli et al., 1999). Cryopreservation of sperm in suspension or in intact tubuli and testis biopsies should be avoided as the products of lysis after thawing may be toxic to sperm quality. No evidence for a 'protective' effect of including Sertoli cells with spermatids has been reported. Moreover, Sertoli cells have been shown to have DNA damage (demonstrated by TUNEL assay) after freeze-thawing and culture (Antinori et al., 1996; Gianaroli et al., 2000; Tesarik et al., 2000). For immature cells, continued spermatogenesis is limited after thawing when compared to outcomes with fresh samples. It is noteworthy, however, that Steele et al. (2000) reported no detectable change in DNA integrity after thawing of testicular spermatozoa. Although clinical studies with thawed testicular spermatozoa are limited in number, outcome efficacy has been reported to be either inferior or comparable to ICSI cycles in which fresh testicular spermatozoa were used (Verheyen ET al., 1997; Holden et al., 1997; Friedler et al., 1998). Pregnancies with thawed spermatids, including those matured in vitro after thawing, have also been reported (Antinori et al., 1996; Gianeroli et al., 2000; Tesarik et al., 2000).

Several techniques specific for testicular sperm samples exist and were developed to address the occurrence immotile spermatozoa after thawing, which can develop motility after a brief period of in vitro culture (Edirisinghe et al., 1996; Nijs et al., 1997). In addition to the acquisition of motility, putative viable spermatozoa can be identified by the occurrence of osmotic shock in the hypo-osmotic swelling (Casper et al., 1996). Enhanced motility can also be encouraged by exposure to pentoxyfiline which stimulates mitochondrial metabolism and therefore motility (Sai et al., 2000). Where very few motile spermatozoa exist, collection in a 'host' zona pellucida for cryopreservation has been suggested (Walmsley et al., 1997; Hsieh et al., 2000) such that even a single spermatozoon may be available for ICSI after thawing. The efficacy of this procedure remains to be determined.

SAFE CRYOPRESERVATION OF SPERM

Cryostorage of spermatozoa may be required for extended periods and therefore, both the documentation and method of preparation must be carefully controlled. A signed and executed patient consent form must define

the length of storage (usually five years). Costs involved, and disposition in the case of death or divorce. . Prior to cryopreservation, all patients should be screened for infectious diseases such as hepatitis A, B or C, and HIV as good medical practice in general, and because of suggestions of viral transmission in liquid nitrogen (Tedder et al., (1995). Indeed, these investigators reported the transmission of the hepatitis B from a contaminated liquid nitrogen tank. It is recommended that 6-month period of quarantine be followed by a second viral screening before storage in a tank with other samples. When straws are used, the type and method of filling are important determinants of safety and storage Benifla et al. (2000) clearly showed the importance of using cryovials or straws with human spermatozoa by demonstrating that the HIV virus was able to leak from and enter unaffected straws. PVC straws cannot be used if they have been sterilised by irradiation as they become toxic. Glass vials, PETG and PVC straws can break or explode when cooled or warmed and consequently can cause leakage in liquid nitrogen tanks. Ionomeric resin straws should be used as they remain flexible and can be heat-sealed to ensure a safe non-leaking system. The labelling system to identify straws should be low temperature resistant and preferably located inside the straw as common marker labels may not be permanent. The storage of samples should be monitored periodically and include manual and/or automatic monitoring of the liquid nitrogen levels, scheduled disinfection and inventoried on a yearly basis.

ICSI: WHICH SPERMATOZOON TO SELECT?

ICSI bypasses most of the molecular and cellular events involved in sperm penetration (see Scott, this volume). For this procedure, a single sperm is selected by empirical or biochemical methods (see Kovacs et al, this volume), immobilized by touching the midpiece with a micropipette, followed by aspiration into the pipette and direct insertion into the cytoplasm. Indications for ICSI are listed in figure 6.3 (Nijs and Ombelet, 2000). For ICSI, the selection should not be a random but rather consider motility, morphology and other markers of viability.

MOTILITY AND VIABILITY

Motility is a good parameter for viability with ejaculated spermatozoa and preference should always be given to these sperm. Although successful Fertilization and embryo development can occur with immotile ejaculated spermatozoa, the implantation rate however is very poor. The same observation applies to immotile epididymal spermatozoa. In contrast, immotile testicular spermatozoa have been reported to have the same fertilizing potential as their motile counterparts (Nijs et al., 1996), and their culture in vitro is often accompanied by the acquisition of 'sluggish' tail

movements which may be associated with additional maturation. If immotility persists for all spermatozoa after extended culture, it is suggested that the HOS test be applied in order to identify those immotile sperm whose plasma membranes are intact (Ved et al., 1997). As noted above, exposure to pentoxyfylline may also initiate or improve motility (Hurtoda de Mendoza, 2000). However, both techniques are can be technically complicated and at present, positive outcomes have been limited.

MORPHOLOGY

The morphology of the spermatozoon is an important but not absolute parameter for the selection of a healthy spermatozoon. Some head, tail and midpiece abnormalities can be linked to the genetic content of the spermatozoon that is being selected and should therefore play an important role in the sperm selection prior to ICSI. Heterologous ICSI combined with genetic studies correlates the morphology of the spermatozoon to its genetic content. Lee et al. (1996) showed that 26% of the spermatozoa with *amorphous, round or elongated* heads have structural chromosomal anomalies. The majority of *large* headed spermatozoa are diploid (Yurov et al., 1996). *Round headed* spermatozoa seem to have variable deficiency in the component (s) responsible for activating the oocyte after injection (Rybouckin et al., 1997). A heterologous injection into mouse oocyte can identify their possible oocyte activation power. Franken (1998) correlated poor sperm morphology with loosely packed chromatin. Soreng et al. (1998) found a higher incidence of sex chromosome aneuploidy in morphologically normal spermatozoa from patients entering an ICSI program. Ruyu et al. (2001) identified a 2 to 5% aneuploidy rate for chromosome X, Y or chromosome 18 in morphological normal spermatozoa. These results demonstrate that normal morphology is not an absolute indicator for the selection of genetically normal sperm. Hence, observed pregnancy failures among ICSI patients may in part be due to the selection of aneuploid sperm. Ideally, a morphological, genetic and structural study of sperm samples allocated for ICSI should proceed every ICSI attempt to identify those patients with an increased risk for genetic abnormalities and/or poor prognosis in ICSI.

MATURITY

Immature sperm cells recovered from testicular cell suspensions can be used for obtaining successful fertilization and pregnancies after ICSI (Nijs and Vanderzwalm, in press, Kahraman et al., 1976). Their competence is determined by the stage of maturity with normal fertilization and pregnancy rates obtained with elongating, elongated and mature spermatids. Round spermatids and spermatocytes have a lower Fertilization rates (< 20% of the MII oocytes) and less than 4% of the patients become pregnancy (Balaban et

al., 2001). Moreover, progression to the blastocyst stage occurs at a much lower and slower rate in embryos derived from testicular round spermatids. The overall quality of these blastocysts is poor and no spontaneous hatching is observed in vitro (Balaban et al., 2000; Urman et al., 2002; see Viega, this volume). It has been suggested that imprinting disorders in round testicular spermatids may be a significant negative factor in the establishment of competence (Manning et al., 2001). The use of spermatids has also been correlated with a high incidence of major malformations in ICSI cycles (Zech et al., 2000), and this should be of concern if ICSI with spermatids is contemplated. No clear parameters are present for the identification of normal round spermatids (Vanderzwalmen et al., 1998) and the use of such sperm must be considered experimental at present. However, for immature testicular spermatozoa, morphological features such as cytoplasmic retention indicate a lower maturational status and a higher risk for disomy (Ergur et al, 2002; Kovanci et al., 2001; Kocacs et al, this volume) and therefore should be avoided.

With this review of the ‘nuts and bolts’ of sperm analysis, cryopreservation and current selection schemes for ICSI, we have attempted to provide routine procedures which should be recognized by all ART programs, especially those employing IVF or ICSI in their treatment protocols. Regardless of origin or state of maturity, the availability of a small number of spermatozoa may be considered by some to be appropriate for ICSI. We suggest however, that this approach must be carefully evaluated and may be unwise in certain instances. While IVF and ICSI have become first line treatments in many infertility programs, we suggest that this may be unwarranted if a detailed semen analysis and appropriate methods of cryopreservation indicate and provide, respectively, competent spermatozoa in numbers sufficient to use ‘low tech’ procedures such as IUI. While the endpoint for ART procedures is a normal baby, the findings presented in this review indicate that the husband may not always be the best source of gametes to achieve this goal.

Failure of Fertilization with IVF**Agglutination****Severe oligozoospermia –cryptozoospermia****Severe asthenozoospermia (Kartagener *)****Teratozoospermia^o – absolute teratozoospermia -Globozoospermia*****Pinheads*****Immunological factors^o****Obstructive and non-obstructive azoospermia, paraplegia: epididymal or testicular sperm collection****Ejaculatory dysfunction: retrograde ejaculate^o****Impaired spermatogenesis or spermiogenesis: immature spermatozoa**

- **Mature spermatids**
- **Elongating and elongated spermatid**
- **Round spermatids***
- **Secondary spermocytes***

In vitro matured spermatocytes and spermatids***Oncology: Cryopreserved spermatozoa of patients before chemo- or radiotherapy****Diagnostic ICSI: part of the oocytes are inseminated by IVF, part by ICSI
to evaluate oocyte quality at time of oocyte retrieval****Re-ICSI after failed IVF****To avoid polyspermia for patients with a history of repeated polyspermia after IVF****Prior Preimplantation Diagnosis****In vitro matured oocytes*****FIGURE 6.3. Indications for ICSI.**

*Results are very poor with this type of indication (< 30% fertilization rate and/or < 5% pregnancy rate)

^oConventional IVF or IUI can offer acceptable results.

Motility – Viability

Immotile spermatozoa:

- *Totally immotile spermatozoa*: HOS test or PVP treatment for viability check
 - Positive for Testicular spermatozoa: good outcome
 - Positive for ejaculated or epididymal spermatozoa: poor outcome
- *Initially immotile spermatozoa* but motility obtained after culture: good outcome

Morphology of spermatozoon

- *Normal morphology*: 2-5% aneuploidy for X, Y or 18
- *Amorphous head*: 26% have structural chromosomal anomalies
- *Round head*: 26% structural chromosomal anomalies, majority lack oocyte activating factors
- *Elongated head*: 26% structural chromosomal anomalies
- *Large headed*: 90% is diploid

Maturity of spermatozoon

- *Round spermatid*: < 20% fertilization rate, low blastocyst formation rate and <4% pregnancy rate
- *Elongated and elongating spermatid*: normal fertilization and pregnancy rates, high rate of abnormalities in off spring
- *Immature testicular spermatozoa*: normal fertilization and pregnancy rates
- *Immature ejaculated spermatozoa with cytoplasm retention*: risk for aneuploidy, disomy and chromatin immaturity

FIGURE 6.4. Guidelines for selecting sperm in ICSI cycles.

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CHAPTER SEVEN

PREPARATION OF SPERM FRACTIONS AND INDIVIDUAL SPERM WITH LOW LEVELS OF CHROMOSOMAL ANEUPLOIDIES FOR IVF AND ICSI

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INTRODUCTION

This chapter presents an overview of our ongoing work focusing upon the relationship between diminished sperm maturity and the frequencies of chromosomal aneuploidies, and on the efficiency of the various sperm preparation methods utilized in assisted reproduction for elimination of immature sperm. This chapter will also extend to the major new breakthrough of hyaluronic acid (HA) binding by mature sperm for ICSI sperm selection. First we will discuss research carried out by the Huszar laboratory in the past 15 years relating to objective biochemical markers of sperm function and fertility. Subsequently, we will review the current data and will evaluate the relative utilities of swim-up, gradient centrifugation and HA binding for selection of sperm with low levels of chromosomal aneuploidies and high levels of DNA integrity for IVF and ICSI. The key points to be emphasized are the following:

- (1) Cytoplasmic retention as a marker of sperm immaturity, and the two-wave expression pattern of the testis specific HspA2 chaperone protein in developing sperm during meiosis and late spermiogenesis.
- (2) Cellular maturation, plasma membrane remodeling and their contributions to the fertilization function of human sperm.
- (3) The relationship between sperm immaturity and increased frequencies of chromosomal aneuploidies.
- (4) The relative efficiencies of density gradient and swim up methodologies in eliminating aneuploid and diploid sperm.
- (5) The use of HA binding for selection of mature individual sperm with low levels of both chromosomal aneuploidies and DNA degradation.

CYTOPLASMIC RETENTION AND OTHER BIOCHEMICAL MARKERS OF SPERM CELLULAR MATURATION

The primary interest of our laboratory has been the development of objective biochemical markers of human sperm maturity and function which would predict male fertility, independently from the traditional semen criteria of sperm concentration and motility. In measurements of sperm creatine-N-phosphotransferase or creatine kinase (CK), we found significantly higher levels of sperm CK activities in men with diminished fertility (Huszar et al., 1988a; Huszar et al., 1988b). We addressed the reasons underlying the sperm CK activity differences by labeling the enzymatic-active site of sperm CK with ¹⁴C-FDNB followed by autoradiography. In another approach, we visualized the CK in individual sperm with CK-immunocytochemistry (Huszar and Vigue, 1993). The CK immunostaining patterns indicated that the high sperm CK activity was a direct consequence of increased cytoplasmic protein and CK concentrations in the spermatozoon (Figure 7.1). The combination of increased CK and protein concentrations, coupled with the diminished fertility suggested to us that we had identified a sperm developmental defect in the last phase of spermiogenesis when the cytoplasm (unnecessary for the mature sperm) normally is extruded and left in the adluminal area as "residual bodies" (Clermont, 1963).

Upon electrophoretic analysis of human sperm extracts, in addition to the CK-B isoform, we found another ATP-containing protein, which was proportional to the incidence of mature sperm, characterized by low CK-activity and no cytoplasmic retention in semen samples (Huszar and Vigue, 1990). We have recently identified this developmentally regulated protein as the 70 kDa testis expressed chaperone protein, which in man is called HspA2 (Huszar et al., 2000). The close inverse correlation between the proportions of sperm with cytoplasmic retention and ratios of HspA2, indicated that cytoplasmic extrusion and the commencement of HspA2 synthesis are related developmentally regulated spermiogenetic events (In three independent studies the correlation between HspA2 levels and CK-activity was $r=-0.69$, -0.71 and -0.76 , $P<0.001$, $N=159$, 134 , and 119) (Lalwani et al., 1996; Huszar et al., 1992; Huszar et al., 1994).

Before we isolated and characterized HspA2, we assumed that, due to its electrophoretic properties and ATP-content, it was an unusual form of sperm specific CK-M isoform (several properties also indicated that it was not a conventional CK-M, ref 5). Nevertheless, HspA2 proved to be a most useful objective biochemical marker. We have shown that mature and immature sperm differ with respect to degree of cytoplasmic retention, HspA2 ratio, as expressed by the concentrations of sperm CK and HspA2 [$\% \text{HspA2}/(\text{HspA2} + \text{CK-B})$], morphological and morphometrical attributes, zona pellucida-binding properties and fertility (Huszar and Vigue, 1993; Huszar et al., 1994; Gergely et al., 1999). Furthermore, we have established that in

spermiogenesis, simultaneously with cytoplasmic extrusion and the commencement of HspA2 synthesis, the sperm plasma membrane also undergoes a maturation-related remodeling. This remodeling step facilitates the formation of the sites and receptors for zona-binding and for hyaluronic acid binding in mature sperm. We, as well as another laboratory, have also shown that immature sperm have increased rates of lipid peroxidation (Huszar and Vigue, 1994; Aitken et al., 1994). Immature sperm also exhibit higher frequencies of chromosomal aneuploidies. Finally, we established that all sperm maturational events related to the decline of CK activity and increase in HspA2 expression are completed by the time the sperm enter the caput epididymidis (Huszar et al., 1998).

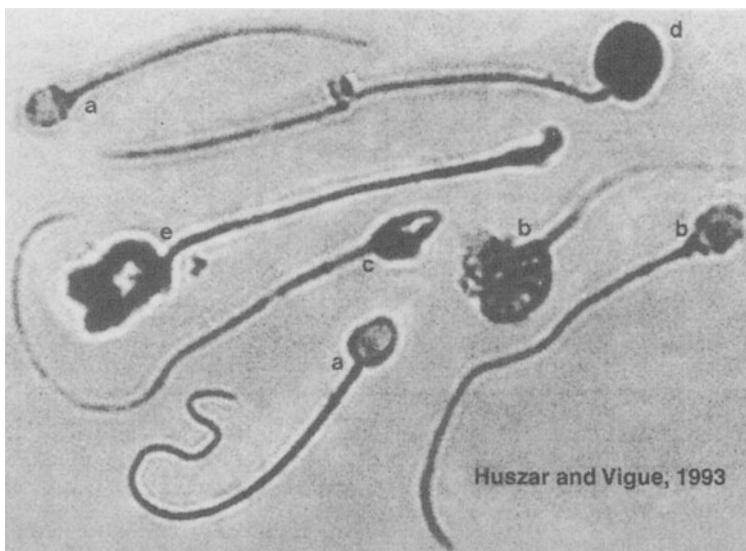


FIGURE 7.1. Montage of CK immunostained sperm, showing the different patterns: (a) Normal sperm with lightly stained or clear heads; (b) sperm with different degrees of CK stippling; (c and d) sperm with various levels of solid CK staining; (e) amorph sperm.

CELLULAR MATURATION AND DEVELOPMENT OF FERTILIZATION FUNCTION

The predictive value of CK-activity, representing cytoplasmic retention, was tested in couples with oligospermic husbands treated with intrauterine insemination. In spite of identical sperm concentration and motility parameters in husbands of couples that did or did not achieve pregnancy, there was a four-fold difference in sperm CK activities ($p<0.001$, those with pregnancies had the lower CK-activity). Also, a logistic regression analysis

indicated that sperm CK activity, but not sperm concentrations, contributed significantly to the predictive power (Huszar et al., 1990a). The value of the HspA2 ratios in the assessment of male fertility was tested in two blinded studies of couples undergoing in vitro fertilization. In the first, we classified 84 husbands from two different IVF centers (without any information on their semen parameters or reproductive histories) based only on their sperm HspA2 ratios into "high likelihood" ($>10\%$ HspA2 ratio) and "low likelihood" ($<10\%$ HspA2 ratio) for fertility groups. All pregnancies occurred in the "high likelihood" group. No pregnancy occurred in the "low likelihood" group. In the "high likelihood" group, if at least one oocyte was fertilized, indicating the lack of oocyte defects in the wife, the predictive rate of HspA2 ratio for pregnancy was a very high 30.4% per cycle. An additional important utility of the HspA2 ratio became apparent: 9 of the 22 "low likelihood" men were normospermic but had diminished fertility. Thus, the HspA2 ratio provided, for the first time, a diagnostic tool for unexplained male infertility (infertile men with normal semen) (Huszar et al., 1992). More recently, we reexamined the utility of HspA2 ratios in predicting IVF failure in 119 couples treated at Yale (Ergur et al., 2002). Similar to the 1992 study, none of the 25 men with $<10\%$ CK-M ratios achieved pregnancy, whether they had low or high sperm concentrations. The value of sperm CK studies has also been confirmed by other laboratories (Gomez et al., 1996; Orlando et al., 1994; Sidhu et al., 1998).

To identify the steps of the fertilization process, at which the low HspA2 immature sperm are deficient, we explored human sperm-oocyte binding. With the study of sperm-hemizona complexes, we established that only the clear headed (low CK), mature sperm were able to bind to the zona pellucida (Huszar et al., 1994). Sperm with retained cytoplasm were deficient in the oocyte binding site (Figure 7.2). In a further study, we confirmed that plasma membrane remodeling occurs in human sperm, simultaneously with cytoplasmic extrusion, during spermiogenetic maturation. This was demonstrated by the close correlation ($r=0.8$) between CK concentration or the HspA2 ratio and the density of the sperm plasma membrane-specific enzyme, β 1,2,-galactosyltransferase in sperm fractions of various maturities (Huszar et al., 1997). This finding well explains two major characteristics of sperm with diminished maturity: cytoplasmic retention and deficiency in zona pellucida binding.

In general, chaperone proteins facilitate the assembly and intracellular transport of proteins. Indeed, the expression of HspA2 is simultaneous with major sperm protein movements underlying cytoplasmic extrusion and remodeling of the human sperm plasma membrane (Huszar et al., 2000). This in turn facilitates the development of the zona pellucida-binding site. We believe that retention of the cytoplasm, and the lack of zona-binding sites in immature sperm are likely related to diminished expression of HspA2, and also to diminished DNA integrity, as a consequence of the impaired delivery

of DNA repair enzymes during and following meiosis. In order to confirm our finding regarding the expression of HspA2 during terminal spermiogenesis, we also examined the expression pattern of HspA2 in human testicular tissue (Figure 7.3). Varying low levels of immunostaining were evident in spermatocytes and spermatids, reflecting the presence of HspA2 in the synaptonemal complexes, but the staining was particularly striking in the cytoplasm of elongating spermatids and mature sperm about to be released from the adluminal compartment. This pattern is consistent with the biochemical data, which indicated that HspA2 is expressed simultaneously with cytoplasmic extrusion in late spermiogenesis (Huszar and Vigue, 1990; Huszar et al., 2000).

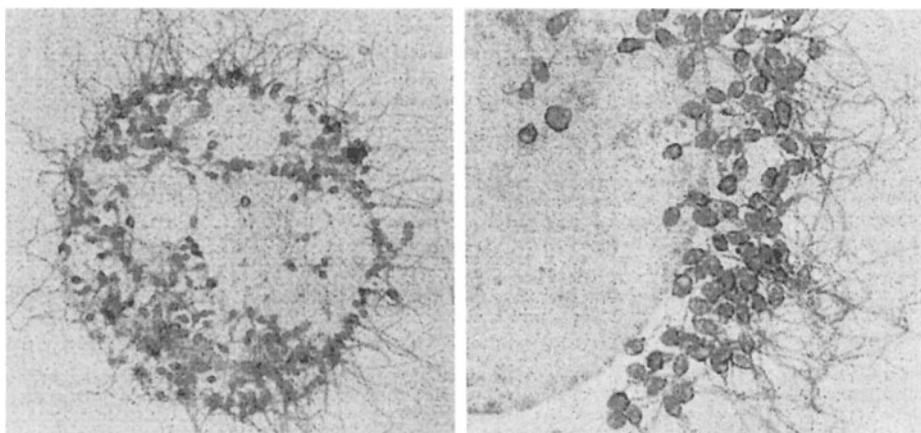


FIGURE 7.2. CK-B isoform immunostained sperm-hemizona complexes. Please observe the lack of dark CK-stained immature sperm with cytoplasmic retention. (Huszar et al., 1994).

From the perspective of male infertility, it is important that synthesis of the HSP70-2 family of proteins is developmentally regulated, and that HSP70-2 appears during meiotic prophase as a component of the synaptonemal complexes. The testis expressed Hsp70-2 protein has been identified in the mouse, and it is first synthesized in pachytene spermatocytes during the meiotic phase of spermatogenesis, and persists in spermatids and mature sperm. The apparent functions of HSP70-2 in mice are maintaining the synaptonemal complexes and assisting chromosome crossing over during meiosis and spermatocyte development (Eddy, 1999). Accordingly, the targeted disruption of the *hsp70-2* gene causes arrested sperm maturation and azoospermia (Dix et al., 1996). These events could be related to faulty meiotic recombination in spermatocytes, to disruption of the meiotic cell cycle regulatory machinery, or perhaps to a more direct disruption of the apoptotic machinery in spermatocytes or even in spermatids or ejaculated immature

sperm. Regarding human sperm, our laboratory was the first to demonstrate the expression pattern of the HspA2 protein in human testis and sperm and to correlate the expression level of HspA2 to sperm function.

RELATIONSHIP BETWEEN SPERM IMMATURITY, DNA INTEGRITY AND CHROMOSOMAL ANEUPLOIDIES

(A) SPERM SELECTION BY GRADIENT CENTRIFUGATION

Because HspA2 is a component of the synaptonemal complex in rodents, and assuming that this is also the case in man, we hypothesized that the frequency of chromosomal aneuploidies will be higher in immature vs. mature sperm (Kovanci et al., 2001). Immature sperm also have a lower density due to retained cytoplasm as compared to mature sperm, which are composed essentially of the nucleus, the acrosome, the tail structures and the plasma membrane. For this reason, mature sperm without cytoplasmic retention sediment in the pellet fraction of well selected density gradients, whereas immature sperm will be retarded in the upper portion, either on the top of the gradient phase or, in multiphase gradients, in the interfaces between the various gradient phases (Huszar and Vigue, 1993; Aitken et al., 1994).

In the study of density gradient fractionation, we have been particularly interested in three issues: (1) Will density gradients eliminate immature sperm from the pellet fractions? (2) Will density gradients remove spermatozoa with chromosomal aneuploidies and diploidies from the pellet fraction? (3) Is there a relationship between the incidences of immature sperm and sperm with chromosomal aneuploidies? It is of note that, in the studies presented below using Percoll gradients, and in other studies both by us and by others, it was established that IsolateTM (Irvine Scientific, Santa Ana, CA) and other improved Percoll derivatives show properties similar to Percoll in sperm fractionation.

In a recent study, we examined these three questions, and the hypothesis that aneuploidies are primarily found in immature sperm (Kovanci et al, 2001). We studied sperm fractions arising from semen and from 80% Percoll pellets of the same ejaculate in 10 moderately oligozoospermic men (concentration: $13.3 \pm 1.4 \times 10^6$ sperm/mL, motility: $50.3 \pm 3.4\%$, all data mean \pm SEM). The selection of this patient population was based on previous work in which we established that men in the lower sperm concentration range, in general, have a higher proportion of immature sperm (Huszar et al. 1988a; Huszar and Vigue, 1990; Huszar and Vigue 1993). Immature sperm with retained cytoplasm, which signify spermiogenetic arrest, were identified by immunocytochemistry. As previous data indicated, the proportion of immature sperm in the ten initial semen was higher than in the respective 80% Percoll fractions ($45.4 \pm 3.4\%$ vs. 26.6 ± 2.2 , medians: 48.2% vs. 25%, $P < 0.001$, $N = 10$ sample pairs).

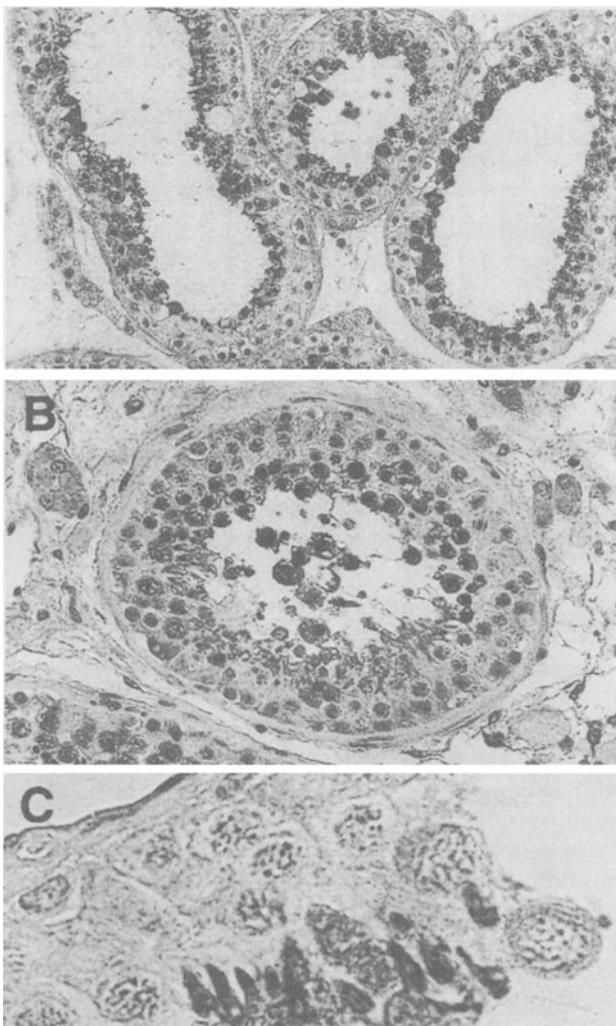


FIGURE 7.3. Human testicular biopsy tissues immunostained with human HspA2 antiserum. Sections A, B and C in the composite represent different magnifications to illustrate the tubular structure, and the staining pattern of the adluminal area. The two-wave expression of HspA2 occurs in the meiotic spermatocytes, and it is more extensive during terminal spermiogenesis in the elongated spermatids and sperm. (Huszar et al., 2000).

Spermiogenesis: Mature and Diminished Maturity Sperm

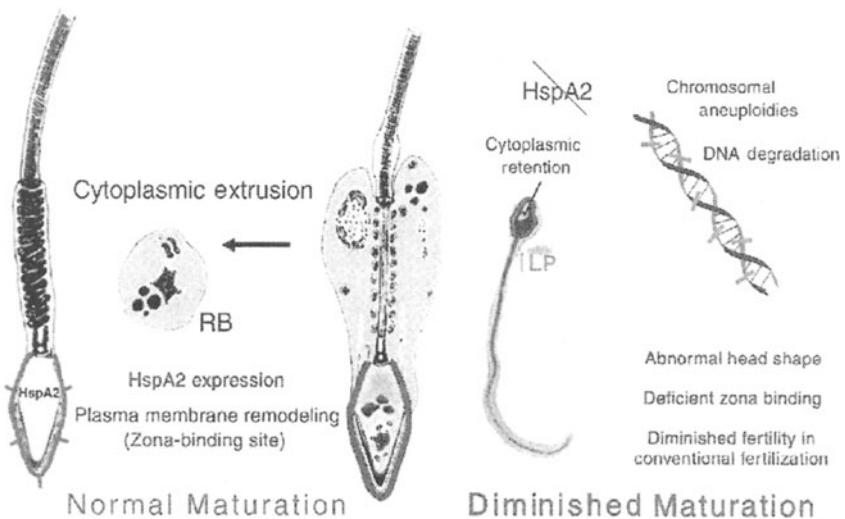


FIGURE 7.4. A model of normal and diminished maturation of human sperm. In normal sperm maturation HspA2 is expressed in the synaptonemal complex of spermatocytes and supports meiosis. HspA2 is probably also involved in the processes of late spermiogenesis, such as cytoplasmic extrusion (represented by the loss of residual body, RB), plasma membrane remodeling, and the formation of the zona pellucida binding side (progression from the blue to the red membranes and stubs). Diminished maturity sperm lack HspA2 expression, resulting in meiotic defects and higher rates of retention of CK and other cytoplasmic enzymes, increased levels of lipid peroxidation (LP) and consequent DNA fragmentation, abnormal sperm morphologies and deficiencies in zona-binding and HA-binding sites. (Huszar et al., 2000).

Using FISH, we evaluated approximately 7000 sperm nuclei in each of the 20 fractions (142,086 sperm in all), using centromeric probes for the X, Y, and 17 chromosomes. In line with the reduction of immature sperm, there was also a concomitant decline in the total disomy and total diploidy categories in sperm fractions from semen vs. 80% Percoll (Table 7.1). There are two major findings: First, there is a significant decline in total disomy (0.54 vs. 0.17%), total diploidy: (0.26 vs. 0.14%) and total disomy and diploidy (0.81% vs. 0.31%, $P < 0.001$ in all comparisons) in the 80% Percoll pellet vs. the initial semen fractions. Second, the man-to-man variations in aneuploidy frequencies are also diminished in the 80% Percoll fractions, since the distributions of values are similar, as detected by the ranges of the point plot (Figure 7.5). These findings are consistent with the enhancement of mature sperm in the 80% Percoll fraction. Also, the 80% Percoll fraction is more homogeneous from the point of view of sperm maturity than the initial samples. In addition, the aneuploidy and diploidy frequencies are reduced to levels (Kovanci et al.,

2001) similar to those in normal men. Further, the total sperm disomy and diploidy frequencies in the initial semen vs. 80% Percoll fractions were reduced 2.8-fold (range: 1.7-4.7). The mean decline in the 10 sample pairs was more distinct in the comparison of disomies (3.3-fold, range: 2.5-4.7) than in the comparison of diploidies (2.1-fold, range: 1.7-3.0). Thus, disomies are more related to the elimination of immature sperm from the semen than are diploidies (Table 7.1).

RELATIONSHIP BETWEEN THE PROPORTION OF IMMATURE SPERM AND FREQUENCY OF CHROMOSOMAL ANEUPLOIDIES

In order to further substantiate a potential relationship between the incidence of immature sperm and of aneuploidies/diploidies, we performed correlation analyses between the proportion of immature sperm with cytoplasmic retention and the frequencies of total disomies, and between total diploidies and total disomies+diploidies, respectively (Kovanci et al., 2001). In line with our hypothesis, the data indicated that there was a close correlation between the incidence of cytoplasmic retention and the frequency of disomies in the 20 samples ($r=0.7$, $P<0.001$). Among the various disomies, the Y disomy correlated best with the incidence of immature sperm in the samples ($r=0.78$). These data further suggested that our hypothesis was correct: the majority of disomies are found in immature sperm with cytoplasmic retention. However, surprisingly, there was no correlation at all between the immature sperm and the incidence of diploidies. ($r= 0.03$); the occurrence of diploidies was not related to sperm immaturity.

B) EFFICIENCY OF THE SWIM UP TECHNIQUE IN ELIMINATING SPERM WTH ANEUPLOIDIES.

The swim-up technique is frequently used for sperm preparation in the methods of assisted reproduction of IUI, IVF- ET and in other approaches in which an enrichment of motile sperm is beneficial. The genetic integrity of sperm in the swim up fraction is important from the perspective of sperm selection methods for assisted reproduction, including ICSI. The introduction of fluorescence in situ hybridization (FISH) with chromosome-specific DNA probes has facilitated the detection of aneuploidies of the X, Y, and several autosomal chromosomes, as well as diploidies. The publications dealing with sperm FISH results, following swim-up separation, have primarily focused upon the question of elimination of sperm with aneuploidies and diploidies in the swim up fractions, but reported results are conflicting.

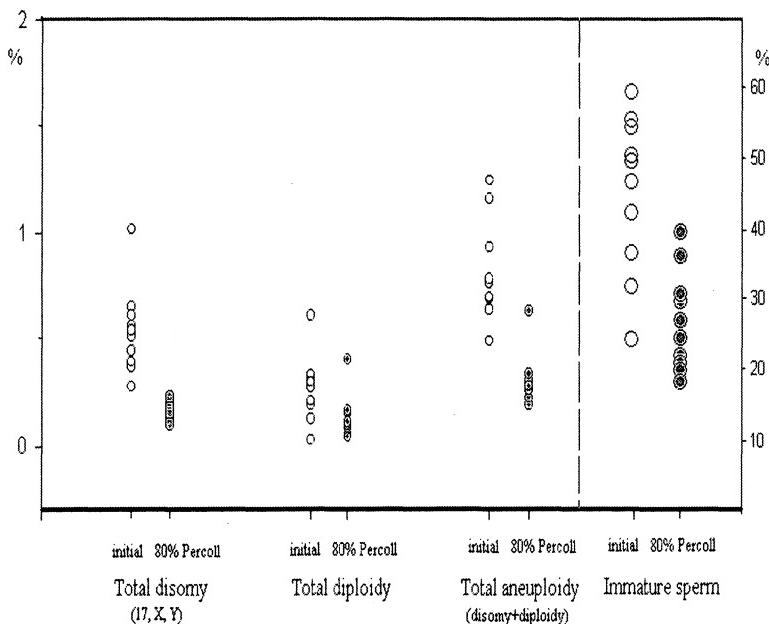


FIGURE 7.5. Distributions of aneuploidy frequencies and the proportions of immature spermatozoa in the initial semen and 80% Percoll fractions of the 10 individuals. (Kovanci et al., 2001).

There are reports which indicate that there were no changes in either the aneuploidy or diploidy frequencies in the initial semen vs. the swim up sperm fractions (Downie, et al, 1997). Yet, in other studies, the authors reported a decline in the frequency of diploidy in the swim up fractions (Han et al, 1994). In another study, the frequencies of diploidy and disomy were examined after separation of sperm fractions by glass wool filtration, by swim-up, and by two-layer discontinuous Percoll gradient centrifugation. The authors did not observe differences in the frequencies of disomies or diploidies between the initial and swim-up sperm fractions by any of the three fractionation methods (Samura et al, 1997).

In another study, the proportion of sperm with disomy and diploidy sperm were monitored in the initial semen and in the swim-up fraction in samples of <30 and >30 million sperm/mL concentration (Li and Hoshiai, 1998). There were no differences in disomy or diploidy frequencies between the initial semen and swim up fractions in the > 30 million/mL samples, but there was a reduction in aneuploid and diploid sperm in the < 30 million/mL group. The Li study was relevant to our work, because, similar to our findings,

Table 7.1. 80% Percoll density gradient fractionation: Frequency of aneuploidies (chromosomes X, Y and 17)

Subject	Sperm Fraction	Disomy X			Disomy Y			Disomy 17			XX Diploidy			XY Diploidy			Total Disomy			Total Diploidy			Total Aneuploidy		
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	Initial semen	0.11	0.06	0.68**	0.17†	0.07	0.04	0.08	0.04	0.08	1.02**	0.20	0.20	1.25**											
	Percoll 80%	0.03	0.01	0.13	0.03	0.04	0.00	0.06	0.06	0.20	0.10	0.10	0.30												
2	Initial semen	0.04	0.17	0.07	0.10†	0.14†	0.07	0.11	0.38†	0.32	0.70*	0.70*													
	Percoll 80%	0.03	0.04	0.08	0.00	0.01	0.07	0.07	0.16	0.16	0.31														
3	Initial semen	0.01	0.15	0.27**	0.08	0.06	0.01	0.06	0.06	0.52*	0.13	0.65**													
	Percoll 80%	0.03	0.04	0.04	0.03	0.00	0.03	0.03	0.14	0.06	0.20														
4	Initial semen	0.07	0.17	0.22	0.11	0.04	0.10	0.06	0.57	0.20	0.77														
	Percoll 80%	0.03	0.04	0.06*	0.06	0.07	0.01	0.04	0.03	0.20**	0.08	0.28**													
5	Initial semen	0.04	0.11	0.24	0.15	0.04	0.06	0.06	0.52	0.55	0.62	1.16													
	Percoll 80%	0.04	0.01	0.08	0.08	0.04	0.04	0.32	0.23*	0.41	0.64**														
6	Initial semen	0.17	0.18	0.20	0.11	0.06	0.06	0.17	0.66	0.28	0.94														
	Percoll 80%	0.03†	0.04	0.04†	0.07	0.00	0.06	0.04	0.18**	0.10†	0.28**														
7	Initial semen	0.11	0.14	0.11	0.08	0.06	0.10	0.18	0.45	0.34	0.79														
	Percoll 80%	0.03	0.00*	0.06	0.03	0.01	0.04	0.06	0.11**	0.11*	0.23**														
8	Initial semen	0.07	0.13	0.03	0.06	0.06	0.04	0.11	0.28	0.21	0.50														
	Percoll 80%	0.01	0.06	0.03	0.00	0.04	0.03	0.10	0.10†	0.17	0.27														
9	Initial semen	0.08	0.08	0.17	0.07	0.06	0.03	0.22	0.40	0.30	0.70														
	Percoll 80%	0.00	0.04	0.07	0.04	0.03	0.03	0.11	0.16*	0.17	0.34*														
10	Initial semen	0.22	0.17	0.17	0.06	0.00	0.01	0.01	0.62	0.03	0.64														
	Percoll 80%	0.06*	0.04	0.10	0.04	0.00	0.00	0.04	0.24**	0.04	0.28*														
Total	Initial semen	0.09	0.14	0.21	0.10	0.06	0.05	0.15	0.54	0.26	0.81														
	Percoll 80%	0.03**	0.03**	0.07**	0.04**	0.02**	0.03	0.09**	0.17**	0.14**	0.31**														
Reduction rate		3.0	4.66	3.0	2.5	3.0	1.67	1.67	3.17	1.85	2.61														

Bold numbers represent significantly different comparisons, † p<0.02, * p<0.01, ** p<0.001.
N=142,068 sperm evaluated (71,385 in the initial semen fraction and 70,683 in the 80% Percoll fraction)
From Kovanci et al., 2001.

(Figure 7.5), the authors reported a man-to-man variation in the frequencies of sex chromosome disomies, presumably due to variations in the proportions of immature sperm among their subjects.

This lack of agreement among the various studies probably occurred due to the variations in patient selection and experimental design. However, the most likely source of inconsistency is assuredly the low number of sperm nuclei evaluated. For a reliable assessment of aneuploidy and diploidy rates, considering the mean frequency of 0.1% to 0.6%, or 1 to 6 aberrant sperm nuclei per 1000, one should evaluate approximately 6,000-10,000 sperm in each sample. In the 7 papers cited above, only three studies were based on >3000 sperm per sample, and none involved >5000 sperm.

In order to resolve these uncertainties, we initiated a study focusing upon the efficacy of the swim up method in eliminating immature sperm and sperm with aneuploidies and diploidies. We have examined three questions:

- (1) Does swim up eliminate sperm with diminished maturity?
- (2) Does swim up reduce the frequencies of sperm with aneuploidy and diploidy in the swim up fraction?
- (3) Does elimination of these chromosomal aberrations occur with similar efficiency?

The third question was of particular interest because the gradient centrifugation studies indicated that density gradients are more effective in the elimination of sperm with disomies compared to diploidies. The experimental design relied on the observation that both mature and diminished-maturity sperm sub-populations are present in virtually all semen samples. Thus, the proportions of "mature" and "diminished-maturity" sperm in initial semen and corresponding swim-up fractions were studied, similar to the Percoll gradient experiments. Immature sperm were identified by the presence of retained cytoplasm highlighted with CK-immunocytochemistry. Aneuploidies were detected by FISH.

The validity of these studies is further supported by the results of our previous studies: (i) We demonstrated an association, in general, between percentage of immature sperm and degree of cytoplasmic retention in men in the < 30 million /ml sperm density range. Also, in samples with higher incidences of immature sperm there were also higher frequencies of chromosomal aneuploidies (Kovanci et al, 2001). Thus, in the present study, we selected men with sperm concentrations in the lower range, but good sperm motility. (ii) In addition to the FISH and sperm maturity data, we monitored the motile sperm recovery in each sample in order to assure that the sperm fractions studied were representative. (iii) In order to ensure reliable data regarding aneuploidy and diploidy frequencies, we studied a combined number of about 15,000 initial and swim-up sperm in each patient.

PATIENT POPULATION

In this ongoing study, we have investigated 8 men (sperm concentration: $19.7 \pm 3.4 \times 10^6$ sperm/ml, motility: $45.3\% \pm 2.2\%$, all data mean \pm SEM,). We used a swim up method in which sperm are not pelleted onto the bottoms of conical tubes, but onto one-centimeter flat platforms in tubes developed in our laboratory. The platform, which enhances the efficiency of the swim up procedure, originally consisted of dense oil, but for the past 15 years we have used 1 ml epoxy embedding material to create a similar surface in the tubes (Makler et al, 1984). The efficiency of the swim-up is measured by the motile sperm yield (proportion of motile sperm in the initial semen recovered in the swim-up fraction). In this group, the motile sperm yield was $37.4 \pm 4.3\%$ (Table 7.2). Along with an enhancement in motile sperm in the swim-up fractions, the proportion of immature sperm having cytoplasmic retention also declined (Mature vs. immature sperm: Initial semen - 55.5% vs. 45.5%, swim up fraction - 71.75 vs. 28.3%, $p < 0.001$).

In the 8 individual patients, there were no significant differences between the initial semen and swim-up sperm fraction for any one of the disomies studied. However, considering all disomies, there was a significant reduction in sex-chromosome disomies, with an overall 1.4-fold reduction in the cumulative data for the 8 men (Table 7.3). The diploidy frequencies and reductions thereof showed an outcome quite different from that of the disomy frequencies. There was a significant reduction in diploidy frequencies in six of the eight patients, with an approximately three-fold (2.9) decline in diploidy rates (Table 7.4). As we found previously in the gradient centrifugation approach, swim-up also selects against both disomies and diploidies. However, swim up was more efficient in reducing diploidies than disomies. Also, in contrast to the gradient centrifugation approach, there was no correlation between the reduction in disomies and the proportions of immature sperm with cytoplasmic retention. This indicates that sperm maturity and motility are not closely related.

COMPARISON OF GRADIENT CENTRIFUGATION AND SWIM UP IN ELIMINATION OF ANEUPLOID SPERM.

1. The previous work by Kovanci, et al, 2001 (Table 7.1) Demonstrated that, in a group of 10 men, there was a four to five fold reduction in sperm with sex chromosome and autosomic disomies in Percoll pellets as compared with initial semen. Thus, gradient centrifugation in which the density of the sperm plays a role is very efficient in reducing the proportions of disomic sperm, but is not as efficient in removing diploidies.
2. Swim-up is less efficient in removing sperm with disomies than is gradient centrifugation, most likely because sperm swimming efficiency

and maturity are only loosely related (disomy elimination factors in gradients vs. swim up: 2.7x vs. 1.4x). However, substantially diminished maturity sperm may remain in the pellet, as it has been shown earlier that sperm with abnormal morphology have less efficient motility (Overstreet et al., 1981).

3. The swim-up approach is more efficient than gradient centrifugation in removing diploid sperm (diploidy elimination factor in gradients vs. swim up: 1.85 vs. 2.86). This is true for at least two reasons: One, diploid sperm are heavier, and thus the efficiency of their movement is diminished. More importantly, we have recently found tail aberrations among diploid sperm which is likely lead to non-coordinated motion of the tails apparently causes a reduction in the efficiency of progressive motility.

4. It is of note that the frequencies of disomies and diploidies found in our patient population may not be as high as those found in patients who are severely oligospermic. Such patients would not be good models for this swim-up study because, in addition to their low sperm concentrations, they are also asthenospermic. Thus, we studied moderately oligospermic men, and our results are most applicable to oligospermic or asthenospermic men with low-motile sperm concentrations who are candidates for intrauterine insemination or in vitro fertilization. However, our conclusions do not apply to ICSI patients, in whom the chromosomal aberration changes cannot be confirmed because the low numbers of sperm involved prevent the possibility of FISH analysis.

TABLE 7.2. SWIM UP STUDY: CHARACTERISTICS OF THE PATIENT POPULATION

Patient	Concentration (M/ml)	Motility (%)	Swim-up motility (%)	Motile sperm yield (%)
1	10	50	90	52
2	45.5	42.1	92.6	43.4
3	22.3	46.1	76.1	49.5
4	12.2	30.7	30	10
5	32.7	48.4	90	70.8
6	15.8	42.3	75	51.5
7	10.4	43.4	70	15.5
8	8.9	59.2	50	6.6
Total Mean \pm SEM	19.7 ± 4.64	45.2 ± 2.87	71.7 ± 7.73	37.4 ± 8.33

Table 7.3. Frequency of sperm with disomes (X,Y, and17) in the initial semen and in the swim up sperm fraction Probes 17X, and Y (three color FISH)

Patient No.	Fraction	Cell No.	Disomy X	Disomy Y	Disomy XY	Sex disomy	Disomy 17	Total disomy
1	initial	5283	0.038%	0.038%	0.114%	0.189%	0.057%	0.246%
	swim-up	5015	0.040%	0.040%	0.040%	0.120%	0.040%	0.160%
2	initial	7336	0.082%	0.068%	0.082%	0.232%	0.082%	0.314%
	swim-up	7009	0.057%	0.071%	0.071%	0.200%	0.043%	0.243%
3	initial	5032	0.079%	0.020%	0.099%	0.199%	0.139%	0.338%
	swim-up	5012	0.020%	0.060%	0.140%	0.219%	0.040%	0.259%
4	initial	5071	0.118%	0.039%	0.079%	0.237%	0.099%	0.335%
	swim-up	4872	0.062%	0.000%	0.082%	0.144%	0.021%	0.164%
5	initial	5054	0.020%	0.040%	0.059%	0.119%	0.000%	0.119%
	swim-up	5030	0.040%	0.020%	0.060%	0.119%	0.020%	0.139%
6	initial	5146	0.058%	0.058%	0.175%	0.291%	0.350%	0.641%
	swim-up	5087	0.039%	0.020%	0.079%	0.138%	0.197%	0.334%
7	initial	5128	0.059%	0.059%	0.039%	0.156%	0.176%	0.332%
	swim-up	5101	0.059%	0.039%	0.020%	0.116%	0.078%	0.196%
8	initial	5350	0.112%	0.019%	0.037%	0.168%	0.075%	0.243%
	swim-up	5219	0.038%	0.019%	0.000%	0.057%	0.019%	0.077%
Mean ± SEM	initial							
	swim-up							
			1.58	1.29	1.39	1.43	2.11	1.62
	Reduction rate							

Bold numbers represent significantly different comparisons
N=85,745 sperm evaluated (43,400 in the initial semen fraction and 42,345 in the swim-up fraction)

Table 7.4. Frequency of diploidies (X, Y and 17) in the initial and swim up sperm fractions

Patient No.	Fraction	Cell No.	All diploidy %	Cell No.	Dipl. XX %	Cell No.	Dipl. XY %	Cell No.	Dipl. YY %
1	initial	5283	0.208%	5	0.095%	6	0.114%	0	0.000%
	swim-up	5015	0.060%	0	0.000%	1	0.020%	2	0.040%
2	initial	7336	0.164%	1	0.014%	10	0.136%	1	0.014%
	swim-up	7009	0.029%	1	0.014%	1	0.014%	0	0.000%
3	initial	5032	0.417%	4	0.079%	16	0.318%	1	0.020%
	swim-up	5012	0.200%	1	0.020%	8	0.160%	1	0.020%
4	initial	5071	0.414%	10	0.197%	7	0.138%	4	0.079%
	swim-up	4872	0.205%	3	0.062%	3	0.062%	4	0.082%
5	initial	5054	0.158%	1	0.020%	5	0.099%	2	0.040%
	swim-up	5030	0.040%	0	0.000%	2	0.040%	0	0.000%
6	initial	5146	1.593%	7	0.136%	72	1.399%	3	0.058%
	swim-up	5087	0.570%	8	0.157%	21	0.413%	0	0.000%
7	initial	5128	1.404%	41	0.800%	13	0.254%	18	0.351%
	swim-up	5101	0.490%	15	0.294%	2	0.039%	8	0.157%
8	initial	5350	0.953%	26	0.486%	7	0.131%	18	0.336%
	swim-up	5219	0.268%	7	0.134%	6	0.115%	1	0.019%
Mean ± SEM	initial		0.663 ± 0.2%		95.0.228 ± 0.09%		136.0.323 ± 0.15%	47	0.112 ± 0.05%
SEM	swim-up		0.232 ± 0.07%		35.0.085 ± 0.04%		44.0.107 ± 0.05%	16	0.039 ± 0.02%
Reduction rate			2.86			2.68		3.02	2.87

(C) SELECTION OF INDIVIDUAL MATURE SPERM WITH LOW FREQUENCIES OF CHROMOSOMAL ANEUPLOIDIES BY HYALURONIC ACID BINDING

Previously we have found that mature, but not immature, spermatozoa in response to hyaluronic acid (HA) showed increased velocity and retention of long-term motility (Huszar et al, 1990b; Sbracia et al, 1997). We suggested that this effect is receptor mediated. Based on the association between sperm maturation and plasma membrane remodeling, we made the following hypothesis: The exclusive presence of the HA receptor in mature as opposed to immature sperm would permit the use of an applicable HA coated device to facilitate the selection of single mature sperm with low probabilities of chromosomal aneuploidies and high probabilities of intact DNA for ICSI.

The concepts summarized in Figure 7.4 outline the relationships between meiotic defects and lack of plasma membrane remodeling, and between normal meiosis (low levels of aneuploidies) and plasma membrane remodeling during spermiogenesis. These relationships are based on the dual functions of the HspA2 chaperone protein, which apparently supports meiosis as a component of the synaptonemal complex, and facilitates plasma membrane remodeling and the formation of the zona-pellucida and hyaluronic acid (HA) binding sites during spermiogenesis (Huszar et al., 1997, 2000).

The increased rate of chromosomal aberrations and other potential consequences of using immature sperm for ICSI are of major concern. Our new approach for selection of individual mature sperm with decreased frequencies of chromosomal aberrations utilizes the binding of sperm to solid state HA. HA is a normally occurring component of the female reproductive tract.

In ongoing studies, we tested the efficiency of sperm selection with respect to the frequencies of sperm with chromosomal aneuploidies and diploidies. Washed sperm of twelve moderately oligospermic men (OS, sperm conc. \pm SEM: $20.6 \pm 1.7 \times 10^6$ /ml, motility: $54.1 \pm 2.5\%$) were studied. Sperm suspended in HTF were placed over HA spots bonded to Petri dishes (Biocoat Inc., PA). After incubation for 15 minutes, the HA-attached sperm were collected using an ICSI micropipette (Figure 7.6). Aliquots of the sperm suspension and HA-bound sperm were examined after FISH, using centromeric probes for the X, Y and 17 chromosomes. Data were examined by chi-square analysis. In each of the 12 man we analyzed a mean 4500 sperm in the initial sperm suspension, and all HA-bound sperm collected (mean: 753, range: 224-1142 sperm/man). The data indicate that the disomy rates declined in the HA-bound fractions (Figure 7.7). The decrease for sex chromosomes was 4-fold. Diploid sperm decreased 6-fold.

The data indicate that HA selection eliminated sperm with disomy and diploidy with a higher efficiency than either gradient centrifugation or swim up. The 4-fold decline of sex chromosome disomies is consistent with the

increase of chromosomal aberrations in ICSI children. In spite of the sample differences, the aneuploidy and diploidy rates in the HA-bound fraction declined to a narrow 0.04-0.13% range. This is similar to the frequencies reported in sperm bound to the hemizona. Thus, HA sperm selection provides a new, safe and efficient solution for selection of mature sperm for ICSI.

Based on these data, we can conclude that (1) Mature sperm permanently bind to HA coated surfaces. (2) Cellular maturation of the sperm may be tested with HA-binding. (3) The frequencies of chromosomal diploidy are reduced by approximately four to six fold in the HA bound vs. the semen sperm fractions. Further studies in testing the potential relationship between HA- and hemizona-binding of semen samples are also in progress.

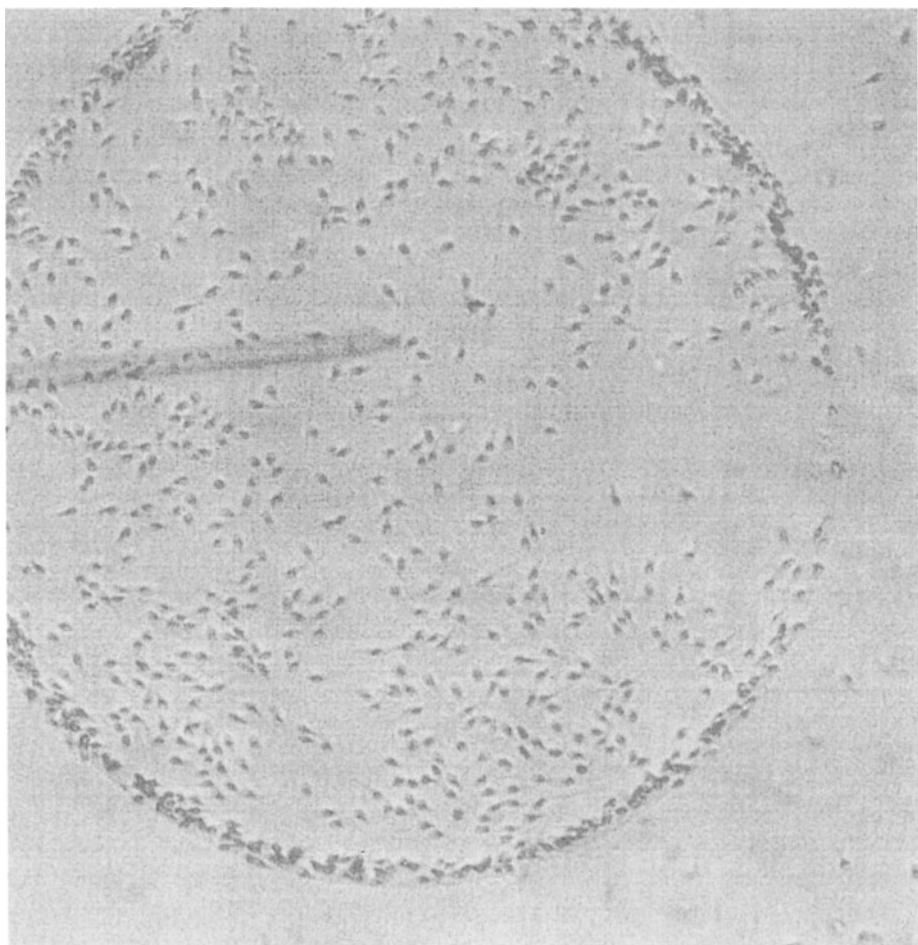


FIGURE 7.6. Removal of HA selected sperm with the ICSI pipette.

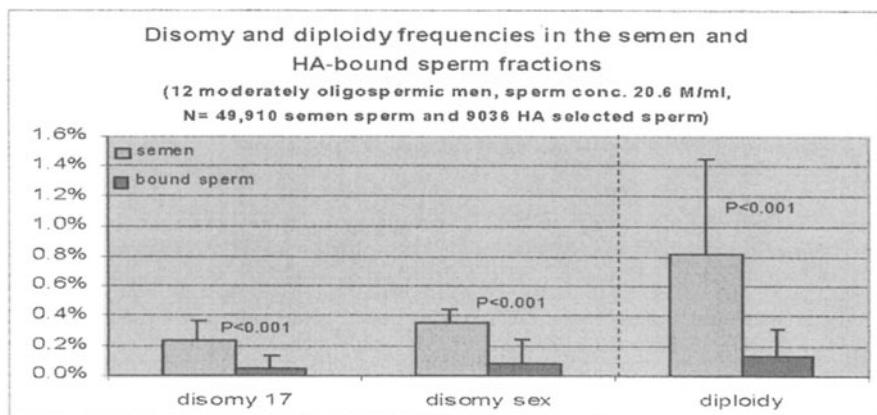


FIGURE 7.7. Disomy and diploidy frequencies in the initial semen and HA-bound sperm fractions.

TABLE 7.5 DISOMY AND DIPLOIDY FREQUENCIES IN THE INITIAL SEMEN AND IN THE HA-BOUND SPERM FRACTIONS

	Disomy		Diploidy
	sex	17	
Initial (%)	0.35	0.23	0.81
HA-bound (%)	0.09	0.04	0.13
Reduction	4.0 X	5.3 X	6.1 X
P (chi-square)	<0.001	<0.001	<0.001

(N=12 oligospermic men)

PERSPECTIVES ON SPERM SELECTION

Our work on the relationship between the proportion of immature sperm and the frequencies of chromosomal aneuploidies (Kovanci et al, 2001) demonstrated that the causal relationship between aneuploidy rates and infertility found in the literature is the consequence of an inadequate definition of male infertility. The conventional definition is based either on the couple's fertility history or on sperm concentration and motility, which in a substantial proportion of oligospermic or even normospermic men, do not reflect the true status of sperm fertilizing potential. The CK-activity reflects

well the mean maturity of a sperm fraction with respect to cytoplasmic retention. CK activity data, however, do not measure the actual maturity of individual spermatozoa, because of extensive sperm-to-sperm variation in degree of cytoplasmic retention within a single semen sample. The incidence of sperm with diminished maturity, which is important with regard to the FISH data for individual sperm nuclei, was determined by evaluating individual sperm via immunocytochemistry.

The relationship between motility and morphology, and the selective elimination of disomic sperm by gradient centrifugation, are well documented by the significant reductions in proportions of sperm with cytoplasmic retention, and by the significant decreases in CK activities in gradient pellets. (Huszar and Vigue, 1993). Thus, the objective biochemical data confirmed our hypothesis regarding the relationship between sperm immaturity and diminished sperm density, which is due to the greater levels of cytoplasmic retention among immature sperm. Thus, in preparation for assisted reproduction, the swim-up method is very efficient in reducing the proportion of diploidies. In contrast, for the reduction of disomies, gradient centrifugation, such as IsolateTM or other Percoll substitutes, maybe the media of choice.

In addition to establishing the respective efficiencies of gradient centrifugation and swim up in removing sperm with chromosomal disomies and diploidies, our findings clarify discrepancies in the literature regarding the efficiency of swim up in removing sperm with chromosomal aberrations. Another important outcome is the demonstration of the differing efficiencies of gradient centrifugation and swim up in the elimination of sperm with disomies and diploidies, respectively.

The role of aneuploid sperm with diminished levels of plasma membrane remodeling, and other deficiencies in fertilizing function, was not important in the setting of conventional fertilization in the presence of the zona pellucida barrier. However, these issues became highly relevant with the introduction of ICSI, a method which overrides the conventional sperm-zona selection process.

Regarding ICSI, it is important that the HA pre-selection of sperm provides an opportunity for fertilization with mature sperm, which should alleviate many concerns related to this method. Furthermore, the use of HA-coated slides will allow the simultaneous testing of each patient with respect to both the proportion of mature sperm and the total number of motile mature sperm in his semen specimen. These data are very important in the evaluation of couples with male infertility, unexplained infertility, and in the determination of assisted reproduction modality that may be optimally used in the treatment of the couple.

[The editors wish to draw the attention of the reader to the work of Barak and colleagues (Hum. Fertil. 2001, 4:99-103) on the use of Hyaluronase for sperm

immobilization in ICSI programs and its potential in the selection of viable sperm for injection.]

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CHAPTER EIGHT

MATERNAL AGE AND OOCYTE COMPETENCE

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INTRODUCTION

There is a continued trend to delay childbearing to advanced maternal ages. Demographics show that in virtually all Western European countries and the United States mean age to child-bearing tends still to increase (Stephen, 2000). An extreme example is the Netherlands where the mean age of women delivering their first child rose from 24.6 years in 1970 to 29.1 years in 1999 (te Velde and Pearson, 2002). Many couples are still unaware of the fact that advanced maternal age is a major factor in female subfecundity and infertility, and that even the best medical treatments cannot postpone menopause and infertility related to the depletion of the follicle pool. In such cases, oocyte donation is the only currently available option. To account for the increased demand of reproductively aged women to conceive a child in their late thirties or over 40 years, preferentially with their own oocytes, it has become a major challenge in assisted reproduction to assess fertility in individual couples, predict competence of individual oocytes from a heterogeneous population of oocytes retrieved from a reproductively aged woman, and counsel and treat older subfertile patients adequately.

For some time there has been a debate on the relative importance of reduced uterine receptivity and lower quality of oocytes in the perimenopausal woman. Experience with similar implantation and pregnancy rates in young and aged patients undergoing IVF with donated eggs suggests that there is only a small effect of advanced age on implantation (for discussion see: Levran et al., 1991; Pellicer et al., 1995; Klein and Sauer, 2001). Most of the effects of ageing on the uterus may be overcome by optimal hormonal supplementation (Rosenwaks et al., 1995; Klein and Sauer, 2001). So far, no effective treatment is available to account for the most

dramatic effect of advanced maternal age, reduced oocyte number, quality and competence.

MATERNAL AGE AND FERTILITY

Age has an impact on oocyte quality and on fertility throughout a woman's reproductive life, and it is now apparent that the age-related decline in fertility already begins in the late twenties (Ziebe et al., 2001) but is most dramatic above 40 years, when more than 50% of couples report difficulties in achieving a pregnancy (Menken et al., 1986). Female age was the most important factor in success rate of IVF e.g. as demonstrated by a large British study (Templeton et al., 1996). Actually, the decline in fertility begins at a period in reproductive life long before there are overt menstrual cycle irregularities and ovulatory dysfunction (te Velde and Pearson, 2002), when there are still a fair number of primordial follicles present in the ovary (ovarian reserve; Faddy and Gosden, 1996; Faddy, 2000). Distributions of age at last birth in a natural 19th century Canadian population practicing no birth control precede age at menopause in a contemporary population by about ten years (te Velde and Pearson, 2002). The period of subfecundity during perimenopause is accompanied by accelerated decay rate of follicles in the ovary, suggesting a correlation between a critically low size of the follicle pool, accelerated follicular atresia and a concomitant decrease in oocyte quality as women approach menopause (Gosden and Finch, 2000; Faddy, 2000). However, populations of oocytes obtained from one patient can differ considerably in developmental competence. Natural conception in women over fifty years demonstrates the heterogeneity within the population to produce oocytes with high quality and competence even at advanced reproductive ages.

IS ANEUPLOIDY ASSOCIATED WITH THE REDUCED DEVELOPMENTAL COMPETENCE OF OOCYTES?

Aneuploidy in human oocytes is one of the major factors contributing to subfertility in aged women, in pre- and postimplantation failure, spontaneous abortion and in the conception of a trisomic child, particularly in women over 40 years (Bond and Chandley, 1983; Jacobs, 1992; Hassold and Hunt, 2001; Fritz et al., 2001). This is supported by data from oocyte donation programmes showing that risk for spontaneous abortion increases with donor age (Levrان et al., 1991), and there is a marked decline in fertility at about 35 years (Stovall et al, 1991). On average, up to about 15-20% of all conceptuses are estimated to be chromosomally unbalanced (Jacobs, 1992; Eichenlaub-Ritter, 1998), and this contributes substantially to the high rate of fetal wastage in our species (Wilcox et al., 1988). While nondisjunction appears to be fairly low at early maternal ages in couples with a normal karyotype, the

aneuploidy rate (calculated from the incidence of trisomy 21) increases **exponentially** with advancing maternal age in women over 35 years with a dramatically increased risk of nondisjunction in oocytes of women over 40 years (Hassold et al., 1980; Hecht and Hook, 1996). In addition to chromosomal imbalance there may be additional factors, which contribute to lower developmental potential of aged oocytes, since the frequency of spontaneous abortions with and without chromosomal imbalance is higher in aged women (Speroloff et al., 1999). Aneuploidy may therefore present the "tip of the iceberg" in the reduction of maturational and developmental competence of the human oocyte. The etiological factors possibly related to increased errors in chromosome segregation at oogenesis and reduced oocyte competence are briefly discussed in this chapter and some current and future options for prediction, prevention and treatments of subfertility associated with advanced age are discussed.

AGE AND CHROMOSOME-SPECIFIC RISKS FOR TRISOMY

According to the concept of an influence of age on oogenesis rather than spermatogenesis, analysis of the segregation and distribution of polymorphic markers in chromosomes from human trisomies showed that the majority of the extra chromosomes are, in fact, of maternal meiotic origin (Hassold, 1998, Hassold and Hunt, 2001). About 90 % of trisomies 13, 15, 21 and 22 possess an extra maternal chromosome and about 80% of all cases result from nondisjunction of the homologous chromosomes at meiosis I (for references see Eichenlaub-Ritter, 2000). Trisomy 16 is exclusively of maternal origin with an error in meiosis I in 100% of all clinically recognized cases (Hassold et al., 1995). Trisomy 18 is also mostly derived maternally but is predominantly associated with a failure in proper chromatid segregation at meiosis II (Bugge et al., 1998). The one exception is the extra X chromosome in 47,XXX or 47, XXY cases, which is paternally derived in 50% of all cases (Hassold, 1998; Thomas et al., 2001). This suggests that chromosome-specific mechanisms exist, which preferentially effect the proper segregation of specific homologues or sister chromatids at female meiosis (Warburton and Kinney, 1996).

Rates of nondisjunction and errors in chromosome segregation in clinically recognized trisomic pregnancies reflect these differences in the susceptibility of individual chromosomes to nondisjunction but also depend on the relative chances of survival of the chromosomally unbalanced embryo. Trisomy data from clinically recognized pregnancies and chromosomal studies in spare and donated oocytes from assisted reproduction show that nondisjunction of trisomy 16 is one of the most common disorders (e.g. Benadiva et al., 1996; Sandalinas et al., 2002). It increases in a near linear fashion throughout advancing maternal age, while trisomy 21 and trisomy of the other small acrocentric chromosomes are low at early reproductive ages and increase

exponentially from about 35 years onwards (Hassold and Hunt, 2001). The risk for multiple trisomies increases even more sharply and exceeds the rate for trisomy 21 in women over 40 years of age (Hassold et al., 1980; Hassold and Hunt, 2001). Large chromosomes of the A and B groups appear to be infrequently involved in the genesis of trisomy (Angell, 1997; Pellestor et al., 2002). Reasons for the differential susceptibility of individual chromosomes to nondisjunction during progressive maternal ageing are not yet understood. The differential susceptibility of individual chromosomes is expected to contribute to heterogeneity in developmental competence between cohorts of oocytes from young and aged but also from individual patients. Chromosomal analysis of spare or unfertilised oocytes from stimulated cycles has not consistently found age-related increases in aneuploidy (for discussion see Plachot, 2001). However, conventional karyotype analysis is difficult in human oocytes, methods involving *in situ* hybridisation are intrinsically error prone and populations of oocytes available for analysis have been extremely heterogeneous (for discussion see: Pellestor et al., 2002). Recent studies employing polar body biopsy (see Verlinsky and Kuliev, this volume) or using donated oocytes and spectral karyotyping, which identifies all chromosomes, support the concept that there is a link between age and increased susceptibility to nondisjunction at oogenesis (Verlinsky et al., 2001; Sandalinas et al., 2002). High rates of aneuploidy, mosaicism or chaotic chromosomal constitutions in human preimplantation embryos suggest that primary as well as secondary nondisjunction are contributing to reduced developmental competence of aged oocytes and embryos (Delhanty et al., 1997; Iwarrson et al., 1999; Harrison et al., 2000; Sandalinas et al., 2001). Accordingly, mathematical modelling based on retrospective and prospective experimental observations on apoptosis in preimplantation embryos predicts that most embryos are already developmentally programmed at the one-cell stage. For example, aneuploidy, deficiency in factors regulating zygotic gene expression at or after compaction, or other intrinsic disturbances rather than culture conditions may effect the fate of the preimplantation embryo and postimplantation survival (Hardy et al., 2001).

PREDETERMINED VERSUS AGE-RELATED RISKS FOR NONDISJUNCTION

High fidelity of chromosome segregation generally depends on recombination between the two parental homologues originally derived from the father and the mother (for discussion see: Smith and Nicolas, 1998). Early stages of pairing and recombination take place in the embryonic ovary when oocytes progress through prophase I of meiosis (Tease et al., 2002). Univalent chromosomes are physically unattached and therefore have a high risk for random orientation and segregation at meiosis I. Transgenic animal models show that failure in expression of gene products required for pairing,

recombination and DNA repair may result in sterility due to meiotic arrest and germ cell death at prophase I of meiosis (Roeder and Bailis, 2000) or as a consequence of high rates of aneuploidy, especially in oocytes (Hunt and Hassold, 2002). Selection against achiasmatic germ cells appears to be dimorphic between the sexes (e.g. Baudat et al., 2000; Hodges et al., 2001; Libby et al., 2002). Feedback mechanisms (checkpoints) leading to meiotic arrest at prophase I when there are pairing or recombination disturbances appear rather permissive in oogenesis (Woods et al., 1999; Tease et al., 2002). In consequence, mutant males in animal models are sterile and do not produce sperm while some oocytes with univalents and errors in chromosome segregation survive to ovulation in females with the same genetic defect (e.g. Woods et al., 1999; Hunt and Hassold, 2002). This dimorphism probably contributes to the gender specific susceptibility to meiotic errors seen in the human and to age-related reduced oocyte competence.

Not only the absence of recombination but also the number and distribution of sites of exchange and chiasmata have an influence on susceptibility to meiotic errors (Koehler et al., 1996; Ross et al., 1996; Smith and Nioclas, 1998). Animal models reveal that even subtle perturbations in recombination and genetic exchange at prophase I of oogenesis within the embryonic ovary dramatically effect the fidelity of chromosome segregation at oogenesis (Yuan et al., 2002). The meiotic origin (meiosis I or II, paternal or maternal origin), the length of the recombination map, and the relative numbers and position of exchanges have been analysed retrospectively in extra chromosomes of trisomies to test for possible correlations between disturbed recombination in early stages of meiosis and aneuploidy in ovulated and fertilized aged oocytes. The recombination map has then been compared with that of "normally" segregating chromosomes. Trisomy 21 derived by a maternal first meiotic error involves predominantly achiasmatic chromosomes at all maternal ages. In addition, particularly those chromosomes 21 having reduced recombination in the vicinity of the centromeres (short recombination map in the proximal parts of the chromosome) have a high risk for nondisjunction at meiosis I although a very distal chiasma may still be present (Sherman et al., 1994). Chromosomes 21 with an excess of recombination in the vicinity of the centromere appear rather predisposed to errors in segregation of chromatids at anaphase II and not at anaphase I as might be expected (Lamb et al., 1996; Petersen and Mikkelsen, 2000). Since chiasmata are already resolved at meiosis I, the errors in segregation of chromatids of chromosomes 21 with excess proximal recombination are probably a consequence of a disturbance at meiosis I (Lamb et al., 1996). Reduced recombination in proximal regions of the extra chromosome in trisomy 16, but normal or even increased recombination in distal parts of the chromosome appears to predispose this chromosome to malsegregation at meiosis I (Hassold et al., 1995). In contrast, trisomy 15 and uniparental maternal disomy 15 (two copies of chromosome 15 from the mother and nullisomy for

the father's chromosome) frequently involve chromosomes with an overall reduced recombination map, and, accordingly, fewer exchanges (Robinson et al., 1998). Predominantly, chromosomes 15 with no or only one chiasma are at risk for nondisjunction in young women. Unexpectedly, chances that chromosomes with two or more chiasmata segregate erroneously from each other during first meiosis of oogenesis increase with increasing maternal age (Robinson et al., 1998). Collectively, these data support the notion that chromosomes with a particular chromosomal history and recombination pattern derived by genetic exchanges at prophase I of meiosis in the embryonic ovary have an especially high chance to fail to be properly processed during the last stages of oogenesis prior to fertilization in an aged female. Globally low rates of recombination appear to be an additional risk factor in trisomy 21 although exchange rates may be still within the normal range (Brown et al., 2000). Since there is no evidence for a general decrease in number or position of exchanges on chromosomes with age (e.g. Lynn et al., 2000), this suggests that there is no "production line" present in the human, which would cause that especially recombination deficient oocytes remain in the ovary until advanced maternal ages. Rather, a "two-hit model" has been proposed suggesting that chromosomes with "susceptible" configuration are present in oocytes at all maternal ages. Such chromosomes segregate with high fidelity in the cytoplasm of a young oocyte but fail to segregate properly within the ooplasm of an aged woman (Lamb et al., 1996). Unlike the situation that prevails in some animals, where univalents and a general reduction in the number of chiasmata may contribute in a predetermined way to age-related errors in chromosome segregation (Henderson and Edwards, 1968; Polani and Jagiello, 1976; Eichenlaub-Ritter, 2000), the reduced competence of human oocytes to faithfully segregate chromosomes at meiosis does not appear to be based on survival of a specific pool of oocytes with predetermined aberrant recombination patterns. Theoretically, it may therefore be considered to "swap" nuclei or cytoplasm between oocytes from young and aged individuals, and thus support normal meiotic chromosome segregation in ooplasm enriched for essential cytoplasmic factors and cell organelles (e.g. mitochondria, see discussion below). Cytoplasmic transfer into human oocytes used for IVF did not have any overt adverse effect on children (Barritt et al., 2001) but is still highly controversial (Cohen, 2002). Transfer of cytoplasm including mitochondria was also used to rescue oocytes from apoptosis (Perez et al., 2000) and may support developmental competence (Van Blerkom et al., 1998). However, especially in view of the consequences of heteroplasmy of mitochondrial DNA and imprinting disturbances for development (see chapters by Brenner and Cummins, this volume), and the still unknown consequences of ageing on chromosomal constitution (see below) more data are required before nuclear or cytoplasmic transfer can be considered in routine clinical application (for discussion see: St. John, 2002).

RELEVANCE OF SPINDLE REGULATION AND KINASE ACTIVITIES FOR AGING OF OOCYTES

Besides of recombination, high fidelity of chromosome segregation depends on the presence of a functional spindle and the spatio-temporal control of chromosome segregation at anaphase of meiosis I and II. Spindle formation requires tubulin polymerisation, activity of microtubule motor proteins of the dynein and kinesin family, and cell cycle-dependent activities of kinases, phosphatases and an appropriate ionic homeostasis (Walczak et al., 1998; Brunet et al., 1998; Lu et al., 2002; Su and Eppig, 2002). In contrast to most mitotically-dividing somatic cells and spermatocytes, which possess two polar microtubule organizing centres (MTOCs) with pairs of centrioles participating in bipolar spindle formation at prophase, oocytes do not contain pairs of centriolar MTOCs but rather have multiple acentriolar MTOCs (Pickering et al., 1988; Battaglia et al., 1996b; Brunet et al., 1998). Chromosomes recruit these MTOCs after GVBD to establish a barrel-shaped anastral spindle with multiple MTOCs assembling at the flat, plate-like spindle poles (Pickering et al., 1988; Kim et al., 1998; Battaglia et al., 1996b; Carabatsos et al., 2000a). Microtubules in oocyte spindles are highly dynamic with rapid de- and repolymerization kinetics and high turnover (Gorbsky et al., 1990). *In vitro* studies using M-phase ooplasm from the frog *Xenopus* or ooplasmic fragments from the mouse containing high activities of maturation promoting factor (MPF) and mitogen-associated protein kinases (MAP kinases) showed that vertebrate ooplasm contains all components to form a bipolar spindle even in the absence of MTOCs or, even in the absence of chromosomes (Heald et al., 1996; Walczak et al., 1998; Brunet et al., 1998; Karsenti and Vernos, 2001). However, motor proteins and high-energy substrates like ATP and GTP are required for polymerisation and for sorting, bundling and organizing spindle fibres (Brunet and Vernos, 2001; Carazo-Salas et al., 2001; Kalab et al., 2002). While antibodies against the motor protein HSET, belonging to the kinesin-like family of microtubule motor proteins, do not severely influence spindle formation, and chromosome alignment and segregation in somatic cells, they interfere with spindle function in mammalian oocytes (Mountain et al., 1999). Local availability of high energy substrates for activity of meiotic kinases and motor proteins is also critical, such that disturbances in mitochondrial number, activity, distribution, and membrane potential might have dramatic effects on the regulation of chromosome segregation.

Expression of Mos-kinase and MAP kinase are needed to maintain proper spindle dynamics and morphology as well as for polar body formation in vertebrate oocytes (Verlhac et al., 1994, 1996 and 2000). Spindle integrity at meiosis II requires also expression of MISS (a MAP kinase interacting and spindle stabilizing protein), which has been identified in mouse oocytes

(Lefebvre et al., 2002). This suggests that oocytes may be uniquely sensitive to compromised function or low expression of such regulatory molecules and gene products, which are required for formation and stability of the anastral spindles.

CONGRESSION FAILURE AS HALLMARK OF REDUCED OOCYTE COMPETENCE

Enhanced polarization microscopy (Polscope) provides a method to analyse non-invasively morphology of the spindle in living human oocytes due to its birefringent properties based on the presence of ordered, paracrystalline bundles of microtubules (Liu et al., 2000b). Oocytes, which do not contain a birefringent spindle, due to absence of a spindle apparatus or unordered spindles fibres have a lower developmental potential as compared to those with visible spindle structure (Wang et al., 2001a,b; Eichenlaub-Ritter et al., 2002). Polscope may be therefore used to assess developmental competence of human oocytes from women approaching perimenopause, and select the best oocyte from cohorts of oocytes obtained from an aged patient (discussed by Eichenlaub-Ritter et al., 2002).

In addition to characteristic disturbances seen with Polscope, immunotubulin analysis of fixed human and mouse oocytes from females of advanced age also revealed frequent failure of chromosomes to congress on the spindle apparatus (the process of alignment of chromosomes at the spindle equator: Eichenlaub-Ritter et al., 1988a,b; Battaglia et al., 1996a; Volarcik et al., 1998). Unfortunately, chromosome alignment cannot be visualised in living oocytes. A mouse model suggests that the disturbances in chromosome alignment and segregation are related to depletion of the follicle pool rather than absolute chronological age of the female (Brook et al., 1984; Eichenlaub-Ritter et al., 1988a). In agreement with this concept, congression failure and aneuploidy are common in oocytes from mutant mice with disturbed folliculogenesis and oocyte growth (Hodges et al., 2002). Marmoset oocytes from small antral follicles mature with a high incidence of spindle and meiotic abnormalities when they are derived from partially naked oocytes of antral follicles (Gilchrist et al., 1995). Therefore, it was speculated that compromised folliculogenesis and disturbed oocyte-somatic cell associations are the basis of spindle abnormalities (Gilchrist et al., 1995). Age-associated disturbances in cell-cell interactions or somatic cell function may cause a loss of coordination between nuclear and cytoplasmic competence and spindle aberrations, unaligned chromosomes and errors in chromosome segregation in oocytes (Carabatsos et al., 2000b; Volarcik et al., 1998; Hodges et al., 2002).

DISTURBED CELL CYCLE AND TIMING OF CHROMOSOME SEGREGATION IN GENESIS OF ANEUPLOIDY

Observations on the chromosomal constitution of unfertilised or donated *in vivo* or *in vitro* matured human oocytes and analysis of maturation kinetics of aged oocytes from a mouse model (Eichenlaub-Ritter and Boll, 1989) suggest that there is a correlation between aberrant meiotic progression and reduced developmental competence. Aged oocytes frequently possess single or pairs of chromatids at metaphase II due to a precocious segregation of sister chromatids at anaphase I or to a premature loss of centromere cohesion prior to anaphase II (Angell, 1997; Pellestor et al., 2002). Unbalanced or balanced predivision poses high risks for meiosis II errors and aneuploidy, and the precocious loss of chromosome cohesion was suggested to be the main mechanism responsible for age-related errors in chromosome segregation (Wolstenholme and Angell, 2000). Studies using fluorescent *in situ* hybridisation (FISH) with chromosome-specific probes in first and second polar body analysis or in chromosomal analysis of unfertilized or donated human oocytes confirm the notion of two mechanisms in malsegregation, but also indicate that nondisjunction of whole chromosomes substantially contributes to aneuploidy in humans (e.g. Volarcik et al., 1998; Clyde et al., 2001; Verlinsky et al., 2001; Sandalinas et al., 2002; Pellestor et al., 2002).

Faithful segregation of chromosomes at meiosis I requires that the sister chromatids of recombined homologous parental chromosomes remain attached to each other all along the chromosome axis, especially distal to the chiasmata, until anaphase I. Resolution of chiasmata at anaphase I depends on loss of cohesion between sister chromatids distal to exchanges, except for the centromeres (Waizenegger et al., 2000). During normal meiosis, cohesion between centromeres of sister chromatids is only lost at anaphase II, similar to mitosis. A complex of highly conserved cohesin proteins found in somatic and meiotic cells mediates attachment between chromosomes (Nasmyth, 2001). A conserved cohesin, which is present in yeast and mammalian meiosis, the product of the *rec8* gene (Watanabe and Nurse, 1999; in humans: *hrec8*; Parisi et al., 1999), is cleaved at meiosis I and II to induce detachment of sister chromatid arms and centromeres, respectively. Upstream of this, proteolysis of other proteins initiating anaphase depends on the activity of APC (anaphase promoting complex), an ubiquitin ligase that also initiates the degradation of cyclin B and thus inactivation of MPF (Buonomo et al., 2000; Nasmyth, 2001). APC is tightly regulated and blocked by a cascade of events triggered by unaligned chromosomes, which are not under tension from spindle attachment to opposite poles (Rieder et al., 1994; Gardner and Burke, 2000; Nasmyth, 2001). Checkpoint control is essential for reductional chromosome segregation at anaphase I (Shonn et al., 2000). The spindle checkpoint delaying anaphase I is expressed late during maturation of mammalian oocytes, and depends on stable attachment of spindle fibres to

chromosomes (Brunet et al., 1999). Since mammalian oocytes may progress to anaphase I in spite of presence of univalents and congression failures (LeMaire-Adkins et al., 1997; Yin et al., 1998a; Hodges et al., 2002), they possibly have a relatively permissive checkpoint. Especially under conditions when oocytes fail to acquire full meiotic and developmental competence in the ovary of an aged woman this poses a risk for errors in chromosome segregation since checkpoints that act as safeguards are possibly permissive.

The turnover, stability and expression of products of genes like *hrec 8* or of proteins, which are involved in the monopolar orientation of centromeres of sister chromatids in homologues at meiosis I (e.g. monopolin, Toth et al., 2000) has not been determined up to now. It is unknown whether prolonged arrest of human oocytes in dictyate stage may eventually lead to a loss of cohesion, initiate precocious chromosome segregation and, thus, in the absence of checkpoint controls, contribute to the high chances for random chromosome segregation in aged oocytes. Precocious separation of homologues has been reported in mouse oocytes aged after ovulation (Mailhes et al., 1998), or delayed in meiosis I by exposure to specific chemicals (Sun et al., 2001). It also occurs more frequently in meiotically delayed oocytes of aged as compared to young female mice (Eichenlaub-Ritter, unpublished). Human oocytes of aged women tend to mature slowly *in vitro* (Volarcik et al., 1998). Especially the short chromosomes of the E and G groups appear to experience predivision in human oocytes (Sandalinas et al., 2002; Pellestor et al., 2002). Currently, more information on the chromosomal status of human oocytes at prometaphase I is required to see whether aged human oocytes frequently carry "functional univalents", as implicated by studies of Angell (1997). Before attempting to substitute aged human oocytes with cytoplasm from young donors, it is essential to know whether originally chiasmatic, precociously-detached univalents are present and will pose risks for errors in segregation, which cannot be compensated by donation of cytoplasmic components.

Unlike any other cell type, mammalian oocytes do express a constitutive meiotic arrest at metaphase II. Arrest is mediated by the activity of cytostatic factor (CSF). CSF arrest depends on activity of *MOS*-kinase and the MAP kinase /Rsk pathway (Maller et al., 2001). Ribosomal p90(RSK) protein kinase activates the spindle checkpoint kinase Bub1 (Schwab et al., 2001). This leads to inhibition of APC required for the metaphase/anaphase transition (Tunquist et al., 2002). Other pathways involved in metaphase arrest probably comprise inactivation of APC by the Mad2 protein, which is retained at the kinetochores of centromeres of misaligned chromosomes in mitosis and is present at aligned chromosomes of mammalian oocytes arrested at metaphase II (Kallio et al., 2000). Aged human oocytes appear to contain less Mad2 mRNA as compared to those from young women (Steuerwald et al., 2001). The Em1 protein is another component inhibiting APC in oocytes (Reiman and Jackson, 2002). Deregulation of this and other cell cycle

regulating gene products might contribute to loss of checkpoint control and precocious detachment and random segregation of chromosomes with advanced age resulting in reduced competence to develop normally after fertilization.

MITOCHONDRIAL FUNCTION, OXYGEN SUPPLY AND SPINDLE DYSgenesis

Mitochondria are especially enriched in the vicinity of the oocyte spindle (reviewed by Bavister and Squirrell, 2000). The sedative diazepam binds to the mitochondrial peripheral benzodiazepine receptor (mPBR) involved in a functional structure designated as the "permeability transition pore" controlling membrane potential and apoptosis (Papadopoulos et al., 2001; West et al., 2001). Diazepam induces disturbances in the association of mitochondria with the spindle in mouse oocytes and also causes meiotic delay or arrest (Yin et al., 1998a; Sun et al.; 2001). This treatment interferes with chromosome congression and causes aneuploidy (Yin et al., 1998a; Sun et al., 2001). Ageing of Leyding cells in males is associated with reduced expression of mPBR, which probably contributes to reduced cholesterol uptake and alterations in steroidogenesis (Culty et al., 2002). Expression of mPBR has yet not been analysed in the aged ovary.

Currently there is a controversy on the involvement of mutations or deletions of mitochondrial DNA (Keefe et al., 1995; Muller-Hockert et al., 1996; Brenner et al., 1998; Barrit et al., 1999; Cummins, 2000; Schon et al., 2000) or on the role of reactive oxygen species (ROS), lipid peroxidation and damage of mitochondria in cell death or age-related reduced maturational and developmental competence of oocytes (Tarin et al., 1998; Van Blerkom, 2000; Perez et al., 2000; Wilding et al., 2001). The efficiency of mitochondrial respiration in oocytes and preimplantation embryos appears closely correlated with the programmed rate of embryo development, and inversely related to maternal age (Wilding et al., 2001). Matters are complex since numbers of mitochondria vary within cohorts of individual oocytes from different patients but also the same individual (Reynier et al., 2001). Mitochondria with high activity and membrane potential possibly occupy distinct sites within ooplasm (Van Blerkom et al., 2002), and their distribution during early cleavage may effect developmental competence (Van Blerkom et al., 2000). Generally, it appears that cohorts of human oocytes with a comparatively high level of ATP appear to have a better developmental potential compared to those with low ATP content (Van Blerkom et al., 1995).

Reduced vascularization and oxygen supply have been implicated in age-related disturbances in spindle formation and chromosome segregation in mammalian oocytes (Gaulden, 1992; Van Blerkom et al., 1997; Van Blerkom, 1998), upstream from mitochondrial dysfunction, and reduced ATP

production. Especially the somatic compartment in the follicle depends on sufficient oxygen supply for oxidative phosphorylation and steroidogenesis. Reduction in oxygen tension during preantral follicle culture severely effects follicle survival and oocyte growth and maturation (Smits et al., 1996). A reduction in ambient oxygen supply after gonadotrophin-induced resumption of maturation of mouse oocytes grown *in vitro* in preantral follicle culture significantly reduces progression to metaphase II. It also disturbs chromosome congression at the metaphase plate (Hu et al., 2001), similarly to what is observed in spontaneously ovulated mouse oocytes matured *in vivo* in females with a depleted follicle pool (Eichenlaub-Ritter et al., 1988a). In contrast to the situation within the follicle, isolated metaphase II oocytes and preimplantation embryos are highly sensitive to elevated oxygen tension and oxidative stress, such that culture in 5% CO₂ in air may cause mitotic nondisjunction (Bean et al., 2002). Mild exposure to peroxide significantly reduces developmental competence of the embryo (Liu et al., 2000a).

Considering that oocyte growth and folliculogenesis may suffer from reduced oxygen supply, especially in patients with a depleted follicle pool, selection for the most highly vascularized follicles appears especially important in optimising treatment for assisted reproduction (Van Blerkom et al., 1997; Gregory, 1998; Huey et al., 1999; Coulam et al., 1999; Bhal et al., 2001). Further studies are needed to assess the predictive value of low/high concentrations of components in follicular fluid that may be related to oocyte competence, like vascular endothelial growth factor (VEGF), insulin-like growth factors, inhibins, activins or angiogenin (e.g. Van Blerkom et al., 1997; Van Blerkom, 2000; Klein et al., 2000; Manau et al., 2000; Quintana et al., 2001; Malamitsi-Puchner et al., 2001; Benifla et al., 2001). The role of mutations or damage of mitochondria in somatic cell function, apoptosis and altered responsiveness and steroidogenesis in follicles resting for extended periods in the ovary has not been analysed but probably contributes to increased cell death of granulosa cells (e.g. Suh et al., 2002). It may, thus, indirectly reduce oocyte competence. Precursor cells of oocytes, primordial germ cells and oogonia, contain a relatively small number of mtDNA templates (the bottleneck) (Shoubridge, 2000), and this may help to protect the oocyte from a high load of mitochondrial insufficiencies (Jansen, 2000), whereas there is no evidence for selection against high levels of pathogenic mtDNA point mutations in oogenesis, in early embryonic development, or in fetal development (Shoubridge, 2000).

ACQUISITION OF COMPETENCE, REGULATION OF EXPRESSION, AND ANEUPLOIDY IN AGED OOCYTES

Mammalian oocytes acquire high maturational and developmental competence in a stepwise fashion during pre-and postpubertal life and during folliculogenesis and oocyte growth. Oocyte growth is a period where

paracrine and autocrine interactions within the follicle orchestrate expression at the transcriptional and translational level (for recent review see Eichenlaub-Ritter and Peschke, 2002; Eppig et al., 1997 and 2002), which equips the oocyte with all factors necessary to support early embryogenesis until zygotic gene activation takes over. Imprinting during oocyte growth (Obada and Kono, 2002) is important in acquisition of competence since it controls development and gene expression from imprinted sites. The low developmental competence of human oocytes matured *in vitro* to metaphase II is associated with the absence of specific proteins in the oocyte (Trounson et al., 2001). Inadequate coupling between oocytes and granulosa cells in aged ovaries could cause similar perturbations in expression in oocytes. Companion granulosa cells appear to play an active role in modulating chromatin condensation and the transcriptional activity in the oocyte, which is required for high developmental competence (De La Fuente and Eppig, 2001; Albertini, this volume). Prolonged inactivation of transcription at the GV stage prior to resumption of maturation interferes with high fidelity of chromosome segregation in mouse oocytes (Zuccotti et al., 1998). Acquisition of maturational competence depends on expression and distribution of cell cycle controlling gene products (Mitra and Schultz, 1996). Continuous cyclin B synthesis is required for maturation to and arrest at metaphase II (Ledan et al., 2001). Age-related impaired interactions between somatic and germ cells can therefore severely influence oocyte fate. Permissive checkpoint controls would potentiate minor disturbances, for instance, in cases where chromosome cohesion is precociously lost. We found that follicular fluid meiosis activating sterol (FF-MAS), which is present in follicular fluid and produced by follicle cells (Byskov et al., 2002), reduced predivision of sister chromatids in mouse oocytes matured to metaphase II without cumulus under sub optimal culture conditions (Cukucam et al., in preparation). Retardation of "cytoplasmic ageing" (Hegele-Hartung et al., 1999) by substances like FF-MAS and optimising culture conditions might therefore be considered as a new approach to retain high developmental competence of human oocytes. As with other methods, further research is needed to critically analyse metabolism and evaluate the influence of carbohydrates, amino acids, growth factors and hormone-like substances like FF-MAS in culture media on oocyte maturation and developmental competence (Downs et al., 2002). Possibly, some of the consequences of ageing like balanced predivision may eventually be overcome by *in vitro* culture of human oocytes under appropriate conditions. Similarly, deleterious effects of the microenvironment on folliculogenesis and oocyte competence may be prevented in follicle culture.

AGE AT MENOPAUSE, REDUCED OOCYTE COMPETENCE AND CHEMICAL EXPOSURES, LIFESTYLE OR POLYMORPHISMS AND MUTATIONS

Depletion of the follicle pool rather than chronological aging is implicated in the reduction in number and competence of oocytes and in predisposition to nondisjunction. Unilateral ovariectomy is not only believed to advanced age at menopause (Hardy and Kuh, 1999), but also to reduce success in IVF (Khalifa et al., 1992). Reductions in pool size, for instance by surgery, may advance risks to conceive a trisomy 21 child (Freeman et al., 2000). Women with a spontaneous trisomic abortion experienced menopause earlier as compared to those with a chromosomally normal birth or aborted foetus (Kline et al., 2000). Smoking, which advances menopause (Kaufmann et al., 1980) and effects oocyte maturation (Zenzes et al., 1997) has been implicated in trisomy 21 with maternal second meiotic origin (Yang et al., 1999). Patients of different age groups with similarly high basal (day 3) serum FSH and estradiol concentrations, which produce only three or less oocytes after routine FSH stimulation have a poor outcome of IVF treatment, similar to their older counterparts (El-Toukhy et al., 2002). Oocytes and embryos from poor responders have increased rates of aneuploidy (Gianaroli et al., 2000).

Reductions in follicular reserve are usually associated with distinct alterations in gonadotrophins and growth factors in serum and follicular fluid (for review see: Soules et al., 2000). FSH levels increase in serum on day 3 of the menstrual cycle, due to the paracrine function of the follicle (e.g. te Velde and Pearson, 2002; Akande et al., 2002; Chang et al., 2002). In effect, there was some evidence that elevated FSH, when related to a depleted ovary, may be a risk factor in Down syndrome (Nasseri et al., 1999; van Montfrans et al., 2001 and 2002). While elevated FSH alone has low predictive value for aneuploidy and reduced competence (Syrop et al., 1999), reduced inhibin B in follicular fluid has served as an additional effective marker of follicular development and has been useful to predict oocyte number and quality of embryos (e.g. Eldar-Geva et al., 2002; Chang et al., 2002). Furthermore, basal E2 levels (<75-80 pg/ml) were predictive of oocyte competence in IVF (e.g. Licciardi et al., 1995). Antral follicle count at early follicular phase was highly predictive, especially, when combined with elevated FSH (Scheffer et al., 1999; Lass and Brinsden, 1999; Bancsi et al., 2002). In three-dimensional ultrasound, total antral follicle number achieved the best predictive value for favourable IVF outcome (Kupesic and Kurjak, 2002). Normo-ovulatory regularly cycling women with a previous poor response to ovarian hyperstimulation for IVF frequently exhibit follicular phase characteristics indicative of ovarian aging (Beckers et al., 2002). Hormonal stimulation cannot compensate for the loss in follicular reserve, so that the poor outcome

of cycles with initial low response to controlled ovarian hyperstimulation cannot be averted by doubling or increasing the hMG dose after 5 days of stimulation (Khalaf et al., 2002). In summary, multivariate analysis of ovarian reserve can help to predict overall oocyte number and competence and outcome in IVF.

Ovarian reserve screening and epidemiological studies on fertility all show that the final stage of reproductive ageing, marked by menopause, greatly varies among women (te Velde and Pearson, 2002). Apart from potential influences of environment, specific exposures or chemotherapy, which may cause premature ovarian failure (POF), the age at natural menopause appears to be predominantly determined by an interaction of multiple genes, e.g. those influencing the size of the founding follicle population as well as the rate of attrition. Torgerson et al. (1997) showed a positive correlation of menopause among mothers and daughters, and twin studies also suggest a strong genetic factor in determination of age at menopause (Snieder et al., 1998; Treloar et al., 1998). Consequently, a woman with a family history of early menopause risks early reproductive failure herself (de Bruin et al., 2001). Apart from reserve screening, identification of genetic determinants of pool size and factors, which influence follicle and oocyte survival and recruitment, and thus, possibly also risk for nondisjunction and reduced oocyte competence will become of considerable interest in the future. There are up to now only few genes identified which influence oocyte survival and aneuploidy, or primordial follicle numbers, recruitment and survival, and age at menopause.

Depletion of ovarian follicles in the human foetus occurs through intrinsic mechanisms of apoptosis in oocytes, and later in adult life the survival of growing follicles may be primarily determined by granulosa cell apoptosis (Vaskivuo et al., 2001). Disturbances in pairing and recombination during early oogenesis within the embryonic ovary are known to cause meiotic arrest, severe depletion of pool size and aneuploidy in the few remaining oocytes with low developmental competence (reviewed by Hunt and Hassold, 2002). In animal models, homozygous mutations in genes in recombination frequently result in sterility, in spite of the survival of some germ cells (Hunt and Hassold, 2002). It may be expected that alleles with a more mild phenotype exist and could contribute to POF and early risk for aneuploid oocytes with compromised developmental potential. Primary numerical or structural chromosomal aberrations like that in Turner syndrome or gonadal mosaicism for chromosomal abnormalities are associated with sterility or subfertility, as a result of precocious depletion of pool size, although follicles may survive into adulthood (e.g. Hreinsson et al., 2002). Reduction in number of ovarian follicles, either due to X mutations effecting oocyte atresia or a restriction in precursor pool size during development are believed to be the reason for the high risk for recurrent spontaneous abortion in association with chromosomal imbalance in some patients with non-random, skewed X-inactivation (Robinson et al., 2001).

Mutations in genes inducing or protecting from follicular apoptosis are also expected to modulate the reproductive life span, ovarian depletion and oocyte competence. Thus, relative expression of *bax* to *bcl* genes determines germ cell survival in the mouse (Rucker et al., 2000). The genetic basis for a census mechanism by which excess numbers of primordial follicles at birth are detected and removed from the ovary by adulthood or, instead, protected from atresia is still unknown (Flaws et al., 2001). Nitric oxide has emerged as protecting follicles from atresia by inducing heat shock protein, HSP70, and suppressing apoptosis induced by *bax* (Yoon et al., 2002). However, a short-term treatment of patients with L-arginine in stimulated cycles in order to induce nitric oxide and enhance follicular vascularity adversely effected embryo quality and pregnancy rate in a recent prospective study (Battaglia et al., 2002).

Other factors associated with induction of ovarian cell apoptosis include GnRH agonist, withdrawal of estrogen, androgens, interleukin-6, insulin-like growth factor binding protein, and tumor necrosis factor (for references see: Klein and Sauer, 2001). Those factors suppressing ovarian apoptosis comprise gonadotrophins (LH and FSH), growth hormone, insulin-like growth factor-1, interleukin-1 β , and epidermal and basic fibroblast growth factor (for references see: Klein and Sauer, 2001). Recent reports further underline the importance of hormonal homeostasis and expression of functional growth and survival factors in fertility and oocyte competence. Beside leukaemia inhibitor factor (LIF, Nilsson et al., 2002), there is evidence from the rat, that insulin via the insulin receptor helps to coordinate primordial to primary follicle transition at the level of the oocyte, relating diabetes to fertility and possibly, to oocyte competence (Kezele et al., 2002). FSH-R status is an important determinant of ovarian aging in mice such that haploinsufficiency induces accelerated reproductive senescence (Danilovich and Sairam, 2002). In fact, FSH-receptor mutations (Aittomaki et al., 1996) and mutations of the *FOXL2* gene mapping on the X chromosome (Pruett and Zinn, 2001; Harris et al., 2002) have been implicated in early menopause in humans. Aromatase deficiency also results in precocious depletion of ovarian follicles in transgenic mice (Toda et al., 2001).

Anti-mullerian hormone (AMH) appears to prevent primary follicle growth in the mouse (Durlinger et al., 2002), and therefore may be an interesting candidate for prolonging reproductive span. Concentrations of AMH are reduced in aged women, and could serve as a novel marker for ovarian aging and reduced competence (de Vet et al., 2002). Reduced expression of AMH might contribute to the rapid depletion of the pool in perimenopausal women. Other genes with products implicated in ageing or POF when deregulated may also have a link to reduced oocyte competence as a result of depleted follicle pool or aberrant oocyte-somatic cell signalling. Gene chip technology led recently to the discovery of pentraxin 3 (Ptx3 or PTX3), a novel factor induced by GDF-9 in granulosa cells of preovulatory

follicles. PTX3 deficiency causes subfertility due to defects in the integrity of the cumulus cell-oocyte complex (Varani et al., 2002).

Aggressive chemotherapy can cause POF, and the chromosomal constitution and competence of surviving, ovulated oocytes coming from partially depleted pools in women treated with a gonadotropin-releasing hormone agonistic analog is unknown (Blumenfeld, 2002). The significance of diet, life style and environmental exposures for oocyte competence with advancing maternal age still needs to be analysed. High dose oral contraceptives slightly but significantly reduce age at menopause (de Vries et al., 2001). This may be the reason for the additive effect of contraception and smoking in the risk for trisomy 21 in association with second meiotic nondisjunction in oocytes (Yang et al., 1999). Low weight at two years was associated with relatively early menopause, highlighting that early as well as later life influences may effect ovarian ageing, and stressing the importance of investigating factors from across the life course in their significance for oocyte competence (Hardy and Kuh, 2002).

In conclusion, tests on follicular reserve as well as analysis of family history, predisposing polymorphisms, mutations or life style and environmental factors will become useful in the future to predict oocyte competence in women approaching perimenopause, or younger patients who experience premature ovarian failure associated with precocious depletion of the follicle pool.

COMPETENCE, MORPHOLOGY AND CHROMOSOMAL CONSTITUTION

Pronuclear scoring (Montag and van der Ven, 2001) and polar body biopsy screening for aneuploidy (Verlinsky et al., 2001) are two methods, which can be employed to select for competent oocytes/embryos of women approaching menopause in countries where ethic and legal restraints prohibit preimplantation genetic diagnosis. Aneuploidy as most important etiological factor in reduced competence does not induce a consistent aberrant morphological feature in the aged oocyte or its embryo to be used in assessment of competence (Rosenwaks et al., 1995; Janny and Menezo, 1996). While monosomy of autosomes appears to effect survival and development of the preimplantation embryo, trisomy cannot be recognized by dysmorphology, and more than one third of all trisomic embryos may reach the blastocyst stage (Sandalinas et al., 2001). Absence of checkpoints sensing chromosomal aberrations (Harrisson et al., 2000) and sensitivity of the preimplantation embryo to mitotic nondisjunction under suboptimal culture conditions (Bean et al., 2002) can contribute to mosaicism and reduced developmental competence of aged fertilized oocytes after IVF. Extensive mosaicism was detected in blastocysts derived from aneuploid embryos

(Ruangvutilert et al., 2000; Sandalinas et al., 2001) and in blastocysts with normal morphology (e.g. Delhanty et al., 1997). Therefore, prolonged culture does not appear useful to select against chromosomally aberrant embryos. Evenly sized blastomeres may be the only morphological parameter that was reported to relate to a normal chromosomal constitution in embryos derived from donated oocytes of 25-37 years old patients, which were analysed by FISH with probes for the most common trisomies (Ziebe et al., 2001).

SUMMARY AND PERSPECTIVES FOR FUTURE STUDY

The number and developmental competence of oocytes decrease with advanced maternal age. However, risks for aneuploidy in oocytes and reduced quality appear to be related to physiological age of the ovary, depletion of the follicle pool and nearness to menopause rather than chronological age. Age at menopause is mainly genetically determined and varies considerably. Therefore, multivariate tests for ovarian reserve and response to stimulation can be helpful in assessment of chances for achieving a pregnancy in IVF in individual patients. Further identification of genes, which modulate age at menopause, will hopefully contribute to predict age-related risks for reduced oocyte competence, and to counsel patients on the prospects of IVF with their own rather than with donated oocytes. At the cellular level, reduced developmental competence of aged oocytes is primarily based on errors in chromosome segregation at oogenesis. Nondisjunction (errors in segregation of whole chromosomes) as well as precocious loss of cohesion between sister chromatids at meiosis I and prior to anaphase II (predivision) contribute to aneuploidy, preferentially involving susceptible chromosomal configurations. Susceptible chromosomes appear to be present in oocytes at all ages but fail to be processed properly in aged ooplasm. While checkpoint mechanisms sensing pairing deficiencies at prophase I and unattached or unaligned chromosomes at metaphase protect against errors in chromosome segregation in spermatogenesis, checkpoints are rather permissive in mammalian oogenesis. Disturbances in spindle formation, insufficient expression of components of checkpoint controls and inappropriate supply of high energy substrates for motor proteins and activity of meiotic kinases, for example due to compromised mitochondrial function and distribution, may thus be especially critical in aged oocytes. Failures in chromosome congression (the alignment of chromosomes at the spindle equator) are characteristic for aged oocytes and those derived from compromised folliculogenesis. It is therefore important to identify the potentially best follicles and oocytes of a cohort in stimulated cycles e.g. by monitoring blood flow and oxygen supply. Provided there is a sufficient number of oocytes in a patient and ICSI is considered, non-invasive polarizing microscopy can be used to image spindles for oocyte selection. Polar body biopsy or preimplantation genetic diagnosis (PGD) of oocyte and embryos have improved implantation rates in aged patients.

Similarly, localisation and morphology of pronuclei, and embryo scoring can be useful for selection, while risks and benefits of prolonged embryo culture are still controversially discussed. Currently, methods to improve competence of aged oocytes in a prospective way to support oocyte growth and acquisition of maturational and developmental competence, are still in an early experimental stage. The success or risks associated with methods like cytoplasmic transfer, nuclear exchange and *in vitro* maturation to improve oocyte-somatic cell interactions and synchrony in cytoplasmic and nuclear maturation events need to be further explored before being employed in routine clinical application. Here promising observations were made but much more information from appropriate animal models is required. Similarly, so far there are no single highly predictive markers for oocyte competence available, for instance, specific components of follicular fluid or cumulus cells. Multivariate methods to determine ovarian reserve have the best predictive value. A wealth of information will become available through molecular screening and transgenic methodologies. Chip technology, targeted gene disruption, and improvement of maturation protocols, are promising to provide new markers and methods to predict and even improve oocyte competence in humans up to an advanced reproductive age. Similarly, discovery of molecules and conditions delaying depletion of the primordial follicle pool have the potential to preserve fertility and competence of oocytes at advanced maternal age.

Currently there is no evidence that hyperstimulation increases aneuploidy in oocytes or causes loss of oocyte competence (Plachot, 2001). In accordance, experiences with repetitive hyperstimulation in oocyte donation from young women imply that there is no adverse influence on oocyte competence (Caligara et al. 2001). The consequences of consecutive and repetitive hyperstimulation to improve chances for natural conception and for treatment in assisted reproduction cycles need still to be investigated in their potential synergistic effects, and thorough analysis of the medical record by the clinician prior to IFV and ICSI appears essential for future risk assessments. Van Blerkom and Davis (2001) described aberrant spindles in oocytes of mice subjected to repetitive superovulation. Interestingly, oocytes obtained from repetitive stimulation did not exhibit aberrations when they matured *in vitro*. Depletion of the follicle pool in reproductively aged women might also create a microenvironment adversely effecting maturation of oocytes within otherwise healthy follicles. High rates of survival and potential for in-vitro maturation, have been reported for human oocytes obtained from stimulated cycles, which were still in GV stage at retrieval. However, these oocytes appear to have a low developmental competence, possibly because they come from compromised follicles that did not properly respond to stimulation (Trounson et al., 2001). The spindles of such oocytes degenerate rapidly (Combelle and Albertini, 2001). Freezing and thawing appear to have an additional deleterious effect on the organization of the meiotic spindle of

human oocytes cryopreserved at both, the GV and MII stages (Boiso et al. 2002). The rate of apoptosis of human immature oocytes cultured *in vitro* was significantly higher in those from older women who were 41 to 50 years old than in those women 21 to 40 years old (Wu et al., 2000), suggesting that follicular environment prior to resumption of maturation may have an impact on human oocyte competence. By supplementing media appropriately and by culture of follicles or oocytes it might become possible to reverse age-effects and obtain euploid oocytes with high developmental potential in the future. By improving cryopreservation methodology, identifying genes and gene products, which are markers of high competence or improve folliculogenesis and oocyte maturation *in vivo* and *in vitro*, it may become feasible to treat aged patients and thus counteract age effects on oocyte competence. Identifying methods, which decrease follicle recruitment and atresia, might prolong reproductive life span. Furthermore, genetic analysis of alleles and polymorphisms modulating age at menopause will aid in counselling and providing the best prognosis and treatment for aged patients in assisted reproduction.

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CHAPTER NINE

GENETIC DIAGNOSIS OF METAPHASE II OOCYTES

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INTRODUCTION

It is well established that the majority of chromosomal abnormalities originate from female meiosis. According to DNA polymorphism studies, these abnormalities derive mainly from meiosis I (Peterson and Mikkelsen, 2000). It is also well known that the rates for the most common trisomies increase with maternal age, probably due to the age-related reduction of meiotic recombination, resulting in premature separation of bivalents and chromosomal nondisjunction. Meiosis II errors also seem to originate from meiosis I, as a result of the increase in the meiotic recombination rate leading to a separation failure of bivalents.

With an advent of IVF it has become possible to study unfertilized oocytes, although the metaphase II (MII) preparations were of poor quality to provide a reliable estimate of the meiotic error rates. This may explain a wide range of chromosomal aneuploidies described in human oocytes, from as low as 8.1% to as high as 54.2% (Plachot, 1997). The quality of preparations was not sufficient not only for the evaluation of structural chromosomal abnormalities, but also for the exact number of chromosomes. The success rate in obtaining the appropriate MII chromosome preparations has been poor even in the most recent studies (Angel, 1997). Because hypohaploidy might be caused by the spreading technique the total aneuploidy rate in MII oocytes was initially evaluated by doubling the number of hyperhaploid oocytes, although higher rates of hypohaploidy, compared to hyperhaploidy could not be excluded. For example, analysis of the high quality chromosomal preparations obtained by turning the meiotic chromosomes into the mitotic ones using a puromycin induced parthenogenetic activation demonstrated that hypohaploidies in MII oocytes are not due to an artifact (DeSutter et al, 1992).

In addition to a wide range of aneuploidies described in MII oocytes, discrepancy was found also in the types of chromosomal abnormalities. Chromosomal anomalies in MII oocytes were believed to originate from the

errors of the whole bivalents as a result of chromosomal non-disjunctions, but recent studies have suggested a significant contribution of chromatid errors in MII abnormalities, showing also the maternal age dependence, in contrast to the earlier studies (Dyban et al, 1996; Angel, 1997; Verlinsky and Kuliev, 2000).

One of major limitations of MII oocyte studies was the missing information about the corresponding chromosome set extruded in the first polar body (PB1). Without such data the karyotype of the MII oocytes could not be reliably evaluated, particularly in the cases of missing chromosomes or chromatids, which should in these cases be found in the corresponding PB1. In fact, no data about PB1 chromosomes were available, except for the discouraging observation that PB1 chromosomes may be recognized only as non-separated disintegrating chromatid pairs, degenerating to the point of fragmentation after extrusion (Rodman, 1971). However, more recent studies showed that PB1 chromosomes are uncountable only immediately after extrusion, becoming recognizable and countable after 2-3 hours of in vitro culture and during the next 2 to 3 hours, with the following degeneration 6-7 hours after extrusion (Verlinsky and Kuliev, 1993). PB1 chromosomes are still difficult to analyze by spectral karyotyping because of the observed chromosomal overlap, but the whole chromosome-specific fluorescence probes or those specific to chromosomal segments appeared to be useful for testing of chromosomal aneuploidies and translocations (Munne et al, 1998; Verlinsky and Kuliev, 2000).

We introduced an approach for a direct study of the outcome of the meiosis I using PBI, extruded as a result of maturation of oocytes in a standard IVF procedure (Verlinsky and Kuliev, 2000). To investigate the accuracy of PB FISH analysis for the meiotic error prediction, we have initially tested MII oocytes simultaneously with their corresponding PBI, using fluorescent alpha-satellite DNA probes for chromosomes 18 and X (Vysis) (Dyban et al, 1996). The normal pattern of fluorescent signals for each chromosome in MII oocytes and PB1 was demonstrated to be paired dots, suggesting that a lack or addition of one or both signals in either MII oocyte or PB1 represents a direct evidence of the first meiotic division error. Because both PB1 and corresponding MII oocytes were studied simultaneously, any missing or extra signals in PB1 were confirmed to reflect an exactly opposite pattern in the corresponding MII oocytes, suggesting a high accuracy of PB1 testing for prediction of the MII oocyte genotype. Similar results were reported with the addition of the chromosome 13/21 probe (Daily et al, 1996).

Based on these data, PBI testing was applied clinically for pre-selection of aneuploidy free oocytes in IVF patients of advanced maternal age (Verlinsky et al, 1995; 1996; 1998a). However, these data showed, that the genotype of the resulting oocytes could not be accurately predicted without the information about the outcome of the second meiotic division, which could be inferred from the study of the second polar body (PB2) (see below). Our

present experience includes more than one thousand clinical cycles involving FISH analysis of approximately 6733 oocytes (Verlinsky et al, 1998b; 1999;2000;2002), which demonstrates the accuracy of preselection of aneuploidy-free oocytes by testing PB1 and PB2.

POLAR BODY REMOVAL – AN ACCURATE APPROACH FOR GENETIC DIAGNOSIS OF METAPHASE II OOCYTES

Polar body removal (PBR) is performed following hormonal hyperstimulation and oocyte retrieval using a standard IVF protocol, as described in detail elsewhere (Verlinsky and Kuliev, 2000). Following the retrieval each oocyte is transferred to a micromanipulation dish with a drop medium containing sucrose. Then the oocyte is secured by the holding pipette and oriented using the microneedle to visualize PB1 at the 6 o'clock position. Using the microneedle the opening is made in the zona pellucida at the 4-5 o'clock position by rubbing the microneedle against the holding pipette. The aspirating blunt micropipette (15-16 μm in diameter) is then passed through the opening to PB1 and gentle suction is applied to aspirate PB1 into the micropipette. Pressure from the hydraulic system is equilibrated prior to withdrawing the aspirating micropipette to avoid any damage to the oocyte.

The oocyte is then washed in IVF medium, inseminated with motile sperm, or using ICSI, examined for the presence of pronuclei and extrusion of PB2, which is then removed in the same way as PB1. The oocyte is rotated to position the opening at the 5 o'clock with PB2 in focus and the micropipette is advanced to PB2. Using gentle suction PB2 is aspirated into the micropipette and the procedure continues as for PB1 removal. Oocytes are returned to culture dishes and observed for cleavage next morning.

A sequential PB1 and PB2 removal is used for testing of single gene disorders, which also involves ICSI, to avoid a contamination with sperm. In contrast, a simultaneous PB1 and PB2 removal is applied for testing of chromosomal abnormalities, which may be fixed and analyzed by FISH on the same slide, with no risk for mixture of the results of each PB. The procedure of simultaneous PB1 and PB2 removal is similar to that performed for PB1 (Verlinsky and Kuliev, 2000).

The biopsied oocytes are returned to culture, checked for cleavage, and transferred, depending on the genotype of the corresponding oocytes. The embryos resulting from unaffected oocytes are transferred back to the patient within the implantation window, or in the next cycle, if oocytes were frozen at the pronuclear stage. Accordingly, the oocytes predicted as affected are used for follow up analysis by polymerase chain reaction (PCR) or FISH analysis to confirm the PB diagnosis.

Because PB1 and PB2 are extruded from oocyte in a normal process of maturation and fertilization their removal is not expected to have any biological role in the development of embryo. We followed-up the oocytes

after PB1 and PB2 removal through different stages of development and have demonstrated the lack of any detrimental effect of PB1 and PB2 removal (Verlinsky et al, 1992; Verlinsky and Kuliev, 1993)). There was no significant decrease in fertilization rate for oocytes, or cleavage of the resulting embryos following PB1 removal. The percentage of embryos entering cleavage was similar in biopsied and non-biopsied oocytes. There was also no increase in the percentage of polyspermic embryos. Data on the long-term effect of the procedure, inferred from culturing the embryos to the blastocyst stage, demonstrated that the proportion of embryos reaching the blastocyst stage was similar to that known for non-micromanipulated oocytes. A follow-up study of the viability of the resulting embryos through implantation and post-implantation development also suggested no detrimental effect, as the procedure has already been applied in more than 7000 oocytes in PGD for the age-related aneuploidies and single gene disorders. No deleterious effect was also observed in the follow up study of more than two hundred children born following PB sampling (Strom et al, 2000). Similar data were obtained in a study of a possible effect of PB2 sampling (Strom et al, 2000; Kaplan et al, 1995).

CHROMOSOMAL ABNORMALITIES IN METAPHASE II OOCYTES INFERRED FROM FIRST POLAR BODY ANALYSIS

Using specific fluorescent probes for chromosomes 13, 16, 18, 21, and 22, we studied 6733 oocytes, obtained from 1297 IVF cycles (Kuliev et al, 2003). As shown in Table 9.1, 41.7% aneuploidy rate in MII oocytes was detected, as inferred from FISH analysis of the corresponding PB1. On one hand, the observed rate should be an overestimate, as the average maternal age of the IVF patients, from whom the oocytes were obtained, was 38.5 years. On the other, this may be an underestimate, because 3630 of these oocytes were tested using tri-color probe specific for chromosomes 13, 18 and 22, with only remaining 3103 tested using five-color probe specific for chromosomes 13, 16, 18, 21 and 22. The aneuploidy rate in the latter group of MII oocytes was increased to over 50%, but almost half of these abnormalities were due to complex errors, with the involvement of more than one chromosome (Kuliev et al, 2003). Although the rate of aneuploidies may further increase with the study of additional chromosomes, this will probably result in the increase of complex abnormalities, which should not lead to considerable increase of the overall rates.

As can be seen from the types of the errors shown in Table 9.2, the majority of abnormalities in MII oocytes are represented by chromatids errors (63.5%), in contrast to the expected chromosomal nondisjunctions, suggested by most of the previous studies mentioned. However, we still observed chromosomal errors in 6.4% of oocytes, which does not support one of the

recent reports, claiming that all abnormalities in MII oocytes are of chromatid origin (Angel, 1997). So, probably both chromatid and chromosomal errors are involved in producing MII abnormalities, with the frequency of chromatid errors being much higher than chromosomal ones (chromatid/ chromosome abnormality ratio 9:1 in our data). There is no doubt that both of these meiosis I errors lead to aneuploidy in the resulting embryos, as demonstrated by the

TABLE 9.1 SUMMARY OF FISH ANALYSIS IN THE FIRST (PB1) POLAR BODIES*

PB1		
FISH Data	No.	%
Normal	3399	58.3
Abnormal	2432	41.7
Total	5831	100

*(Kaplan et al, 1995; Verlinsky et al, 2000; unpublished data)

follow up study of the embryos resulting from aneuploid MII oocytes, the transfer of which were avoided (Verlinsky et al, 1998b). However, differences in the effect of chromatid and chromosomal errors on the pre- and post-implantation development cannot be excluded, as unfortunately the embryos resulting from these oocytes were neither transferred, nor further cultured, but were used for confirmation of PB1 diagnosis instead.

It is also of interest that at least three times higher frequency for missing compared to extra chromatids in PB1 was observed (48.1% and 15.4%, respectively). The same correlation was observed for missing chromosomes (5.9% and 0.5%, respectively), suggesting a possible maintenance of the extra chromatid or chromosome material in MII oocytes, which is in agreement with a higher frequency of trisomies over monosomies in pre- and post implantation embryos. Overall, of 2432 MII chromosome errors, only 387 (15.9%) were monosomies/ nullisomies compared to 1313 (54%) trisomies/disomies. Although the observed excess of missing signals in PB1 may be attributable to technical errors, such as hybridization failure, it is also possible that a meiosis I checkpoint mechanism exists, preventing an extra chromosome material extrusion into PB1 if meiotic errors occur during the oocyte maturation process.

TESTING FOR MEIOSIS II ERRORS IN EVALUATING NUCLEAR NORMALCY OF OOCYTES

The above data show, that as much as 41.7% of abnormal oocytes may be detected by testing the outcome of the first meiotic division, using PB1 analysis. However, as seen from Table 9.3, 39.5% of these oocytes (965 of 2432 MII oocytes with chromosomal abnormalities) appeared to be abnormal

TABLE 9.2 TYPES OF ERRORS IN THE FIRST MEIOTIC DIVISION*

Types of Errors	Number	%
Extra Chromatid	375	15.4
Missing Chromatid	1169	48.1
Extra Chromosome	12	0.5
Missing Chromosome	144	5.9
Complex	732	30.1
Total	2432	100

***(Kaplan et al, 1995; Verlinsky et al, 2000; unpublished data)**

also following the second meiotic division, as inferred from PB2 testing (Verlinsky et al, 2000;2001;2002). As many as 1077(52.7%) oocytes became abnormal only following the second meiotic division, and could be missed if testing were limited to PB1, suggesting that in order to identify all oocytes with chromosomal abnormalities, the outcome of both the first and second meiotic divisions should be studied, using PB1 and PB2.

In contrast to MII oocytes, there was no difference in the frequency of missing or extra chromatid errors following the second meiotic division (Table 4). Overall, 2042 (35.2%) of 5808 oocytes tested had meiosis II errors, of which 842 (41.2%) were with extra chromatid, 748 (36.6%) with missing chromatid, and 452 (22.4%) with complex errors. Chromosome-specific pattern of meiotic errors showed that, as expected, the most frequent chromosomes involved in meiotic error were chromosomes 21 and 22 (20.8% and 18%, respectively) (Kuliev et al, 2003). Involvement in meiotic error of

the chromosomes 13, 16 and 18 was approximately two times less frequent (10.7%, 8.6% and 13.3%, respectively), and their error patterns were not identical. Chromosome 16 errors originated predominantly in meiosis II (55.9% meiosis II vs. 28.8% meiosis I errors), in contrast to chromosome 13 and 18 errors, deriving more frequently from meiosis I (47.4% and 61.8% from meiosis I vs. 36.5% and 28.5% in meiosis II, respectively).

TABLE 9.3 TYPES OF ABNORMAL OOCYTES BASED ON FIRST (PB1) AND SECOND (PB2) POLAR BODY FISH ANALYSIS*

Types of Abnormal Oocytes	No.	%
PB1 + PB1	965	27.6
PB1	1467	41.7
PB2	1077	30.7
Total Abnormal	3509	100

(Verlinsky et al, 2000; unpublished data)

A high rate of complex errors observed in MII oocytes, as well as in the oocytes following the second meiotic division, may be explained by the age-related effects on recombination frequency, or spindle formation errors (Eichenlaub-Ritter et al, 2002), also reported to increase with age (Battaglia et al, 1996; Petersen and Mikkelsen, 2000). The most recent data on the molecular mechanisms of cohesion of sister chromatids in meiosis may be also of relevance for understanding the nature of the age-related increase of meiotic errors (Nashmyth et al, 2000; Yuan et al, 2002). Whatever the cause of the observed aneuploidy rates, approximately one third of these aneuploidies were represented by complex errors, suggesting that testing for only five chromosomes, would probably cover the majority of chromosomal abnormalities in oocytes. This is not only because the abnormalities of these five chromosomes are most common, but also because the application of additional chromosome specific probes will probably result in the proportional increase of the complex error rate, rather than the overall increase of chromosomally abnormal oocytes.

**TABLE 9.4 TYPES OF CHROMOSOMAL ABNORMALITIES IN 2042
SECOND POLAR BODIES (PB2) DETECTED IN 5808
OOCYTES WITH PB2 FISH RESULTS***

Types of Errors	No.	%
Extra Chromatid (Disomies)	842	41.23
Missing Chromatid (Nullisomies)	748	37.63
Complex	452	22.14
Total	2042*	100

Overall Chromosomal Error Rate in PB2 – 35.2%
(Verlinsky et al, 2000; unpublished data)

PERSPECTIVES ON POLAR BODY GENETIC ANALYSIS

Genetic diagnosis of MII oocytes allows detection of the majority of abnormalities in the resulting oocytes. Our data show that PB1 testing alone should reduce aneuploidy rate in the embryos at least by two thirds. Despite the fact that approximately one third of these oocytes will be aneuploid also following the second meiotic division, PB1 testing may still sufficiently improve the pregnancy rates in poor prognosis IVF patients, or ICSI patients, applying ICSI selectively to the oocytes with aneuploidy-free PB1. Our data also show a comparable aneuploidy rate following the second meiotic division, detected by PB2 analysis. Because only a half of these abnormalities are detected by PB1 analysis as complex errors, to avoid the transfer of all the embryos resulting from aneuploid oocytes, testing of both PB1 and PB2 is required. As PB1 and PB2 are extruded in a normal process of oocyte maturation and fertilization, their removal and testing may become a useful tool in assisted reproduction practices to identify the oocytes without nuclear abnormalities, which should help in the preselection of oocytes with the highest potential for establishing a viable pregnancy, improving significantly the IVF efficiency.

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CHAPTER TEN

OOCYTE COMPETENCE AND IN VITRO MATURATION

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INTRODUCTION

Despite their central role in clinical in vitro fertilization (IVF), the specific factors that establish physiological competence for the human oocytes are largely unknown and as a consequence, the absence of analytical measures to assess competence is a key factor in the unpredictability of outcome in IVF. The inability readily to distinguish between competent and incompetent oocytes has important consequences for the ‘success rates’ of treatment. Not only may incompetent embryos be mistakenly chosen over competent ones for transfer, but more crucially, patients who lack competent oocytes cannot be identified early in the work-up of infertility assessment so as to allow them to be diverted from the distress of cycles of costly and ineffective treatment.

Oocyte competence is an ill-defined term which broadly means ‘capable of producing an embryo which can develop further in a normal manner’. Thus, immature oocytes, being incapable of normal fertilization, are by definition incompetent, unless or until functional maturation is achieved, as are oocytes which have some form of defect, such as a chromosomal anomaly or a cytoplasmic insufficiency. The competence of oocytes is multifactorial and thus a thorough understanding of the long and complex process of oocyte development is essential in order to make any inroads into the potential causes and consequences of oocyte incompetence.

This chapter specifically addresses oocyte competence in relation to maturation in vitro, where maturation usually refers to the final few hours of oocyte development, which normally occurs within an ovarian follicle prior to ovulation. At this time, several aspects of oocyte development that are critical for competence are known to occur, but they remain poorly characterized. In natural cycles, oocyte maturation takes place between the midcycle surge of pituitary gonadotrophins and ovulation, whereas in stimulated IVF treatments, the gonadotrophin surge is simulated by injection of exogenous hCG. Immature oocytes have not yet achieved competence because their genetic

material remains in diplotene of meiotic prophase I and must condense into chromosomes and undergo a reductional division to progress to and arrest at the second meiotic metaphase. Coincident with nuclear (chromosomal) maturation, molecular and cellular changes at the cytoplasmic level occur and this process of 'cytoplasmic maturation' is absolutely necessary for the acquisition of developmental competence. Up to the gonadotropin (LH/hCG)-induced reinitiation of meiosis that precedes ovulation, the ooplasm is principally organized for 'maintenance,' but as fertilization and subsequent embryogenesis become imminent, it rapidly prepares for these events.

Preovulatory maturation normally occurs within the confines of the Graafian follicle, and seems a highly efficient process because at ovulation, the vast majority of oocytes are believed to be at the correct stage of meiosis and fertilizable. The developmental competence of *in vivo* matured human oocytes is virtually impossible to determine, but it is estimated that at least 50% may be capable of producing normal early embryos (Buster et al, 1985; Formigli et al, 1990), although this figure may be somewhat lower if fertilization and preimplantation development occur *in vitro*.

In contrast, the production of competence oocytes after *in vitro* maturation is relatively unsuccessful in humans (Trounson et al, 1994). In this instance, fully grown but functionally incompetent oocytes are isolated from follicles and cultured for 24-48 hours before fertilization is attempted. Substantial proportions of immature oocytes complete nuclear maturation and progress to metaphase II and are fertilized after injection of a sperm (ICSI). However, experience to date demonstrates high frequencies of retarded early development, embryonic arrest and implantation failure (Edwards et al, 1965a; Moor et al, 1998). While *in vitro* maturation (IVM) has achieved significant success in some mammals to become a routine method in certain commercially important species, it has yet to achieve a similar role in clinical IVF. The absence of competent oocytes after IVM is not simply of academic interest, but rather a potentially important issue from the perspective of the patient and clinician. This approach to infertility treatment has been frequently discussed in the literature and popular media as a potential alternative to conventional IVF because in theory, it should eliminate the need for gonadotrophin stimulation, regular ultrasound and estrogen monitoring, and in practice, should afford more precise scheduling for fertilization and embryo transfer. However, for the promise of clinical IVM to be fulfilled, an understanding of what deficiencies may occur *in vitro* needs to be considered in the context of what is currently thought to be required to generate a competent oocyte *in vivo*.

OOCYTE DEVELOPMENT

SUMMARY OF FOLLICULAR GROWTH

Normally, the complete development of a competent oocyte occurs only in the dominant follicle, which by a several physical and biochemical characteristics progressively distinguishes itself from others within a cohort of follicles that begin to develop with each menstrual cycle. While incompetent oocytes may still arise from a dominant follicle, it is generally assumed that features of the dominant intra-follicular environment have been refined by evolution to optimize conditions that promote the maturation of a competent gamete. Therefore, what are the determinants of competence is a question central to any understanding of the reproductive biology of the human female gamete.

The development of competent oocytes that result in normal embryos is a prolonged process, which may be considered to begin during fetal life when a pool of primordial follicles is formed. Primordial oocytes are stored with meiosis arrested in prophase I, and are unable to develop further unless the follicle grows and provides an environment conducive for continued oocyte development. Although follicles grow in a continuous stream from early infancy until the end of the female's reproductive ability, follicles do not attain ovulatory sizes or produce significant quantities of estrogen during childhood. At puberty, co-ordinated cyclic gonadotrophin stimulation from the pituitary gland initiates oocyte maturation and ovulation at mid cycle and is manifest by the onset of menses. Figure 10.1 provides a diagram of the approximate time scale of follicle growth in humans.

SELECTION AND MATURATION OF THE DOMINANT FOLLICLE

It has been suggested that the follicle selected to become dominant and ovulate is normally the largest healthy follicle with a diameter of 5.5-8.2 mm at the beginning of the FSH-dominated follicular phase (Gougeon and Lefevre, 1983). Its granulosa cells appear most responsive to FSH, and modulation of FSH sensitivity may be achieved by local growth factors, for example, insulin-like growth factor-1 (Adashi, 1993). The dominant follicle begins to synthesise systemically detectable estradiol from the mid-follicular phase. As follicle maturation occurs, the granulosa cells acquire LH receptors in response to FSH stimulation, conferring responsiveness of large follicles, but not smaller ones, to LH. As preovulatory follicular maturation continues, the vascularity of the follicle increases, thereby increasing the delivery of gonadotrophin to the maturing follicle as well as the systemic availability of its products, notably steroids (Zeleznik, 1981).

The preovulatory gonadotrophin surge of LH stimulates oocyte maturation, cumulus expansion and the reinitiation of meiotic maturation which progresses from prophase I to metaphase II (MII) in approximately

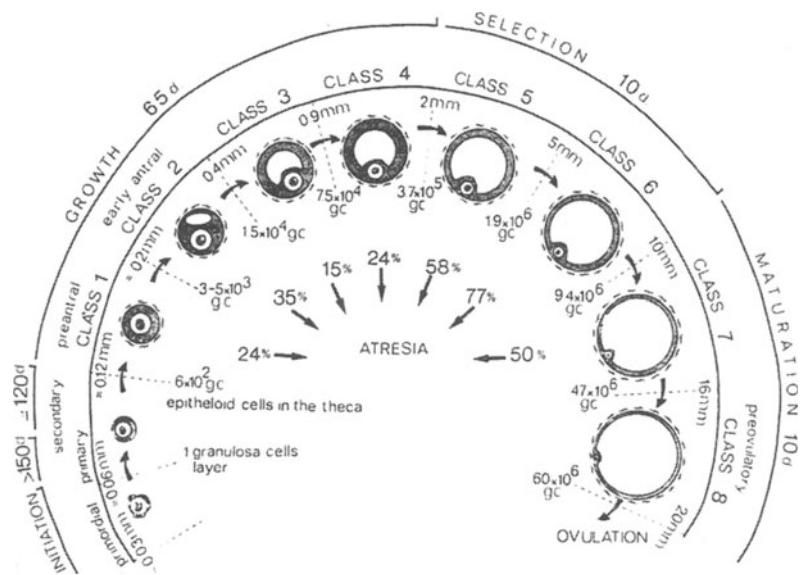


FIGURE 10.1. Stages of folliculogenesis in the adult human ovary and probability of atresia in the eight classes of growing follicles. (reproduced from Gougeon, 1986).

36 hours (Edwards, 1965b). Ooplasmic changes that are coincident with meiotic and characterize cytoplasmic maturation include an increased capacity to release of intracellular calcium (Carroll et al., 1996), alteration of preformed mRNA needed to support embryonic development prior to complete genome activation (Fulka et al., 1998), and a migration to the subplasmalemmal cytoplasm of cortical granules which are involved in the prevention of polyspermic penetration (Ducibella, 1996). Cumulus expansion occurs as a result of hyaluronic acid-rich proteoglycan matrix secretion by the cumulus cells that envelop the oocyte. This expanded matrix holds the oocyte and associated cumulus cells in position and forms forming the so-called oocyte/cumulus complex, whose form is generally thought to facilitate follicular extrusion at ovulation, capture by oviductal fimbria, and sperm penetration and fertilization (Salustri et al., 1996; Matzuk, 2000).

MEIOSIS AND GENETIC MATURATION OF OOCYTES

Meiosis is a unique type of cell division that occurs only in gametogenesis and results in a halving (reductional divisions) of the number of chromosomes and chromatids to enable the reconstitution of the normal adult chromosome number (diploid) after fertilization. Meiosis promotes genetic variation through recombination of chromosomes and corresponding genes. Oogonia in the fetus enter meiosis during the second trimester of pregnancy, but the oocyte arrests in the diplotene stage of meiotic prophase I, only progressing to metaphase II, the stage at which ovulation and fertilization occur, during oocyte maturation in the adult.

During the prolonged arrest at diplotene of prophase I, the oocyte chromosomes have condensed, synapsed with their homologous pairs, undergone recombination, and desynapsed along their lengths, except at the points of crossing over known as chiasmata. Oocytes may remain arrested at this stage for many years (decades in the human) prior to ovulation. The efficient maintenance of this arrested state is therefore crucial to ensure that the stock of oocytes is rationed effectively throughout reproductive life, and that individual oocytes remain stable until the initiation of follicular growth. The surrounding granulosa cells are responsible for oocyte maintenance but the precise regulatory mechanisms involved remain unknown. What is known however, is that the oocytes present in the adult are those which have survived previous waves of atresia that begin during fetal life and result in the apoptotic elimination of nearly 99% of initial oocyte pool.

Before meiosis can resume, arrested immature oocytes undergo significant growth in order to exceed a 'size threshold' for maturation and stage-related changes in chromatin configurations (Combelles et al, 2002). For oocytes to grow, however, growth must also involve the corresponding follicle whose own growth enables the oocyte to produce and store mRNA and protein. The reinitiation of meiosis probably involves a common signaling pathway between the oocyte and its associated granulosa cells, regardless of whether the oocyte is destined to ovulate or degenerate (Barnes and Sirard, 2000). For example, spurious maturation is also observed in degenerating follicles, and may be the result of degenerative changes which uncouple where oocyte/granulosa communication (Gougeon and Testart 1986).

At maturation, the oocyte resumes meiosis I and progresses through metaphase, anaphase and telophase, ejecting the first polar body, a tiny daughter cell containing half the chromosomal complement along with a minimal amount of the cytoplasm. This asymmetric division of ooplasm occurs as a consequence of the peripheral position of the spindle which allows chromosome number to be halved without significant loss of ooplasm. Oocytes progress directly to metaphase II, without replication of DNA or formation of a nucleus, and become arrested again to await fertilization. This

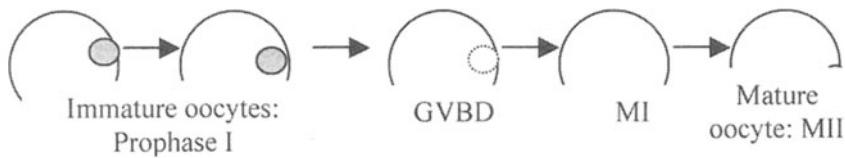


FIGURE 10.2. Diagram of oocyte morphology during maturation

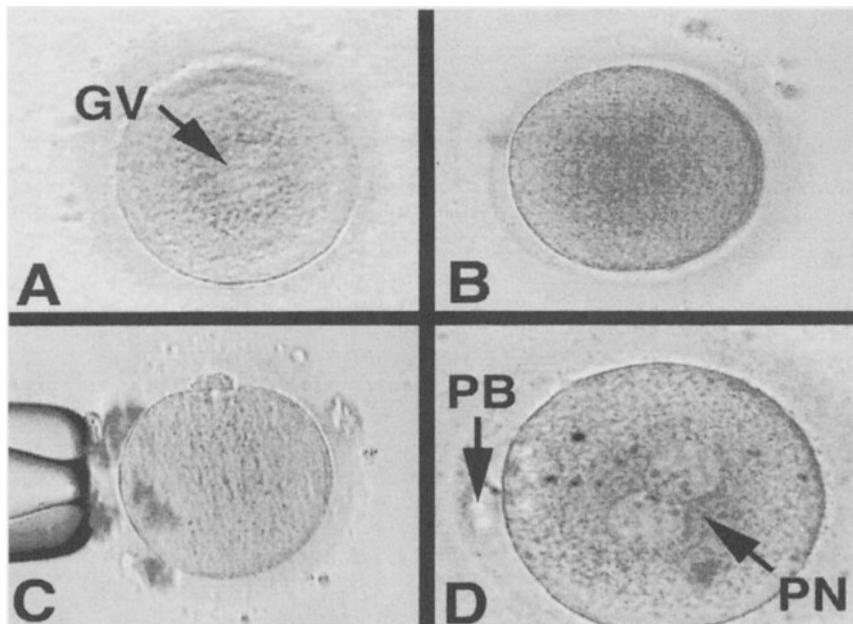


FIGURE 10.3. Stages of human oocyte maturation in vitro. (a) Immature oocyte collected from the unstimulated ovaries of a patient with PCO. A germinal vesicle is visible in the centre of the oocyte (GV) and the oocyte has no surrounding cumulus cells. (b) After one day in culture, the oocyte has undergone germinal vesicle breakdown (GVBD). (c) After two days in culture, the oocyte has one polar body (top of the oocyte as shown) and is presumed to be in MII. It is injected with a single immobilised sperm by the ICSI procedure. (d) Confirmation of fertilization, shown by the presence of two pronuclei (PN) and two polar bodies (pbs, one out of focal plane).

arrest point, where the 23 chromosomes (each two chromatids) are aligned on the spindle, is less stable than prophase I, and oocytes can remain functional and ready to develop normally after fertilization for at most 24 hours (Park et

al., 2000). Meiosis II is resumed only upon sperm penetration or parthenogenetic activation.

Morphological evidence of meiotic resumption is the disappearance of the oocyte's nucleolus and nuclear envelope (the germinal vesicle), by a process termed germinal vesicle breakdown (GVBD). Nuclear maturation is assumed to occur when the polar body is extruded, as illustrated in Figures 10.2 and 10.3.

KEY STAGES OF DEVELOPMENTAL COMPETENCE AND THEIR CONTROL

The nuclear and cytoplasmic programs of oocyte maturation can proceed independently, as described in the following sections, however, full developmental competence is acquired only when the two processes are closely integrated (Moor et al., 1998; Fulka et al., 1998).

NUCLEAR MATURATION

MATURATION PROMOTING FACTOR (MPF)

Many of the proteins that regulate mitosis also regulate meiosis. The G₂ to M-phase transition in fully-grown oocytes is driven by maturation (or M-phase) promoting factor (MPF)- the primary molecule involved in meiotic cell cycle progression (Eppig, 1996; Fulka et al., 1998; Briggs et al., 1999; Trounson et al., 2001). MPF is a serine-threonine kinase protein heterodimer composed of a regulatory subunit, cyclin B and a catalytic subunit p34^{cdc2}. Figure 10.4 is diagrammatic representation of the molecular structure. Cyclin B and p34^{cdc2} become associated, and dephosphorylation of p34^{cdc2} catalyzed by a phosphatase encoded by the cdc25 gene occurs on residues tyrosine-15 and threonine-14 (Gautier et al., 1991; Gabrielli et al., 1992; Izumi and Maller, 1993). Active MPF is required for the initiation of nuclear maturation and the condensation of chromosomes at metaphase I and MPF activity is detectable before or coincident with GVBD. Trounson and colleagues (2001) demonstrated the activation of MPF in IVM studies using human oocytes from unstimulated ovaries, leading them to conclude that the activation of MPF in vitro is similar to the situation that prevails in vivo. Entry into anaphase I coincides with the inactivation of MPF, probably due to proteolytic degradation of the cyclin unit of MPF (Murray et al., 1989). Entry into MII requires a second increase in active MPF, and because c-mos maintains MPF at high levels, cell cycle progression is arrested until fertilization, where sperm penetration results in an increase in intracellular free Ca²⁺ which induces MPF activity and cyclin B degradation which permits the completion of meiosis (Murray et al., 1989).

MITOGEN ACTIVATED PROTEIN KINASE (MAP) KINASE

MAP kinase is a serine-threonine kinase activated via a protein kinase cascade at the onset of oocyte maturation in mouse (Verlhac et al., 1993), pig (Inoue et al., 1995) and Xenopus (Haccard et al., 1990). MAP kinase, although activated as oocyte maturation begins, is not necessarily required for GVBD in mice (Sun et al., 1999b; Trounson, 2001). In human oocytes, p42ERK2 is the main form of MAP kinase (Sun et al., 1999). MAP kinase has not been widely studied in human oocytes, although it is known to be inactive in immature oocytes, active in mature oocytes and shows decreased activity after pronuclear formation (Sun et al., 1999). The pattern of activation of MAP kinase during human oocyte maturation is similar to the one detected in other mammals (Trounson et al., 2001).

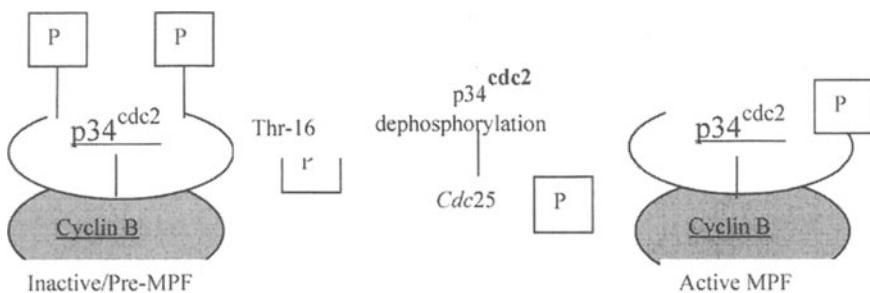


FIGURE 10.4. Diagram illustrating the activation of maturation promoting factor (MPF). (adapted from Eppig (1996) and Trounson et al. (2001).

PURINES

cAMP participates in meiotic arrest and may prevent GVBD by the inhibition of MPF activation or by down regulation of p34^{cdc2} dephosphorylation, and decreases below a threshold level before meiosis can resume. Two other purines, hypoxanthine and adenosine, prevent GVBD in vitro in mouse oocytes by promoting high cAMP levels in the oocyte, and may enter the oocyte via the gap junctions. Hypoxanthine prevents the degradation of cAMP whereas adenosine promotes the generation of cAMP by stimulating adenylate cyclase, acting as a substrate for cAMP production (Eppig, 1996; Briggs et al., 1999). In human oocytes, 6-dimethylaminopurine (DMAP) reversibly inhibits GVBD and cell cycle progression by inhibiting the post-translational dephosphorylation of p34^{cdc2} that triggers MPF activity (Jessus et al., 1991), but does not interfere with protein synthesis (Rime et al., 1989; Fulka et al., 1991; Trounson et al., 2001).

CYTOPLASMIC MATURATION

Cytoplasmic maturation refers to the processes that prepare the oocyte for fertilization, activation, formation of pronuclei and preimplantation development. As with nuclear maturation, competence to undergo cytoplasmic maturation is probably acquired in sequential steps during maturation as the oocyte progresses from the germinal vesicle stage. Most deficiencies in oocytes that effect competence are believed to be associated with defects in cytoplasmic reprogramming during maturation rather than with meiotic progression. The effects of cytoplasmic aberrations are normally manifested during the pre-and peri-implantation stages and present as cellular irregularity, slow cleavage, premature arrest, low cell numbers and implantation failure of implantation (Moor et al., 1998).

PROTEIN SYNTHESIS

Proteins are synthesized in growing oocytes from transcripts encoded by nuclear and mitochondrial genes and depending upon their origin and regulatory properties, may be used for communication with surrounding granulosa cells by the oocyte and to support early embryonic development (Briggs et al., 1999). Oocyte mRNA translated in the oocyte is stored in a stable, dormant form until GVBD (Bachvarova, 1985). When compared with in vivo matured MII oocytes retrieved from stimulated ovaries, a reduced protein content was detected in in vitro matured MII human oocytes derived from unstimulated ovaries (Trounson, 2001). Certain proteins were not detected in oocytes matured in vitro, probably including molecules essential for cell cycle regulation and normal embryo development (Trounson, 2001). If confirmed, this is an important finding as it could indicate specific molecular defects associated with IVM that precludes the establishment of normal developmental competence.

INOSITOL LIPIDS AND CALCIUM

Sperm binding and fusion promote Ca^{2+} -dependent changes that result in oocyte activation. Ca^{2+} is released from intracellular stores via an IP_3 -dependent signal transduction mechanism (Homa, 1995). The oocyte's capacity to release intracellular Ca^{2+} is relatively low during the early stages of maturation, but reaches a maximum at MII (hamsters; Fujiwara et al., 1993). The release of Ca^{2+} is essential for oocyte activation and pronuclear formation and normally coincides with fertilization (Eppig, 1996). Defects in the ability of the oocyte to respond to signals that mobilize Ca^{2+} or during cytoplasmic maturation result in inadequate Ca^{2+} storage or sequestration could indicate developmental incompetence, and may be especially relevant if related to IVM.

GUTATHIONE PRODUCTION

The levels of glutathione in oocytes rise during maturation (Perreault et al, 1988). Glutathione is a reducing agent, which aids sperm decondensation and may have a role in various mammalian species in the formation of the male pronucleus, as well as potentially providing protection from pathological free radical oxidation. (Yoshida et al., 1993; De Matos et al., 1996). Whether levels of glutathione differ between in vitro and in vitro matured oocytes needs to be determined.

COMPETENCE FOR THE RELEASE OF CORTICAL GRANULES

In mature oocytes, cortical granules take up a peripheral position just below the oolemma. The fusion or penetration of a single spermatozoon into the oocyte triggers cortical granule release by exocytosis and blocks polyspermy by reducing the penetrability of the zona pellucida to continued penetration by accessory spermatozoa (so-called zona reaction) (Hoodbhoy and Talbot, 1994) The exocytosis of cortical granules can be induced by Ca^{2+} ionophores and the response of the oocyte to this treatment increases with the progression of maturation (Sathananthan and Trounson, 1982; Ducibella, 1996). Lack of exocytosis due to incomplete cytoplasmic maturation can lead to polyspermy, whereas premature release, possibly due to inappropriate or extended culture, may reduce the permeability of the zona to sperm penetration and hence fertilization potential. (Lopata and Leung, 1988; Ducibella et al, 1995).

FOLLICLE SIZE AND TIMING OF OOCYTE RECOVERY

The size of the follicle from which the oocyte originates influences its developmental capacity both in vivo and in vitro, as critical aspects of developmental competence are acquired during follicular growth (Tsuji et al., 1985). The endocrine milieu that changes through the menstrual cycle may also effect gonadotrophin sensitive follicle growth and possibly other aspects of follicle and oocyte development.

Whitacre et al. (1998a) reported that a significantly higher percentage of human oocytes retrieved from 9-15mm follicles during the follicular phase of the menstrual cycle underwent GVBD than did oocytes collected from follicles that were 3-4mm in diameter during the luteal phase (60% and 48%, respectively). It is possible that follicles <10mm in diameter contain fewer developmentally competent oocytes than do larger follicles common to stimulated IVF cycles (Dubey, 1995), or in the unstimulated or minimally stimulated follicular phase (Trounson et al, 1998). A minimum follicle

diameter of 5mm was reported by Wynn et al, (1998) to be required for oocytes to be capable of in vitro maturation. The establishment of developmental competence may involve important relationships between follicle size thresholds, menstrual cycle timing or endocrine factors that not yet fully understood (Trounson et al, 1998)

OOCYTE SIZE

The human oocyte has a size-dependent ability to resume meiosis and complete maturation, with an oocyte diameter $>115\mu\text{m}$ reported to be necessary for the progression from GVBD to MII (Durinzi et al., 1995), and a better understanding of the minimal requirements for oocyte growth will promote the development of consistent techniques for IVM. Improved understanding of the development of the oocyte is crucial, because the quality of embryos depends on the maternal endowment of RNA and proteins that develops during oogenesis as well as their stability and controlled expression. In this regard, molecular markers of oocyte viability are needed to support current morphological assessments of oocyte and embryo quality. However, the characterization of such markers is not a trivial exercise because clinical use requires that the methods used for their detection be noninvasive nor have iatrogenic effects.

THE CURRENT STATE OF IVM OF HUMAN OOCYTES

It has been known for many years that immature human oocytes removed from large follicles will mature spontaneously in vitro (Pincus and Enzmann, 1935; Edwards, 1965a,b). However, the developmental competence of in vitro matured human oocytes is low (Veeck et al., 1983; Barnes et al., 1996; Coskun et al., 1998), probably due to disruption of the normal follicular control mechanisms regulating this important stage of development (see Albertini, this volume). When oocytes are aspirated from antral follicles early in the follicular phase, follicular and oocyte growth are incomplete, and some follicles may already have initiated atresia. Nonatretic oocytes from such follicles usually require 48 hours of culture to reinitiate meiosis, which is a somewhat shorter longer period as when compared oocytes in the natural cycles where nuclear maturation is initiated by the LH surge (luteinization signal; Cheung et al., 2000). It is possible that maturational and developmental anomalies observed in IVM oocytes are attributable to their truncated growth phase and thus an inability to complete all the necessary transcriptional and translational requirements for maturation and the acquisition of developmental competence.

Following the study of Lonergan et al (1997) who reported improved cattle blastocyst formation from IVM oocytes attributed to temporarily delaying maturation in order to allow for a prolongation of growth, Anderiesz et al

(2000) extended the growth phase of immature human and mouse oocytes *in vitro* with 6-dimethylaminopurine (DMAP), which reversibly inhibits GVBD. It was suggested that DMAP treatment may synchronise nuclear and cytoplasmic maturation, but no evidence of this effect in either mouse or human oocytes was reported. DMAP treatment of human oocytes had no effect on fertilization or development to the blastocyst stage and was found to increase the developmental capacity of mouse embryos. Anderiesz et al (2000) concluded that lengthening the prematuration growth phase by temporarily inhibiting kinase activity with DMAP did not directly improve oocyte developmental competence for the human.

Due to the low developmental competence of IVM oocytes, *in vivo* maturation remains the current method of choice to obtain successful outcomes, despite the need for large doses of exogenous hormones, with their attendant risks and costs (Russell, 1999). This is in contrast to some animal species where IVM is commonly used to obtain viable oocytes for research or commercial purposes without the need for gonadotrophin stimulation (Trounson et al., 1996). Yet, even this method is not without problems, as a proportion of the animals produced are abnormal (Young et al., 1998; Sinclair et al., 2000) as discussed below. One of the present challenges with the human oocyte is to provide optimal conditions for IVM which may require mimicking the microendocrine environment of the dominant follicle as this would seem to be an important prerequisite for immature oocyte to achieve full nuclear and cytoplasmic competence. With IVM oocytes, conditions for fertilization and embryo culture, as well as uterine receptivity at transfer, may be different from conventional IVF cycles, and therefore may need to be differentially optimized if implantation is to occur (Russell, 1998). IVM appears particularly challenging in the human where factors known to have downstream consequences for competence, such as maternal mRNA inheritance, are so poorly understood and difficult to study. Moreover, disruption of these processes have the potential to cause devastating effects upon development not evident by embryo performance *in vitro* during the preimplantation stages.

IVF by its very nature exposes embryos to conditions not normally encountered *in vivo*, and perhaps as a result, unforeseen consequences including the large offspring syndrome (LOS) associated with an increased abortion rate, increased length of gestation, physical abnormalities and increased mortality and morbidity are a result (Walker et al., 1996; Behboodi et al., 1995; Farin and Farin, 1995; Thompson, 1997; Young et al., 1998; Maxfield et al., 1998; Ranilla et al., 1998; Sinclair et al., 1999; McEvoy et al., 2000). Present findings indicate that altered patterns of genomic imprinting are a likely and unanticipated cause of this syndrome (Sinclair et al., 2000; Young and Fairburn, 2000; Khosla et al., 2001).

Although there is no evidence for long-term developmental disturbance associated with human IVF and embryo culture, it is known that oocytes and

embryos up to and beyond blastocyst stage are extremely sensitive to their environment, whether natural or artificial, and their normal development can be threatened in many ways. The extended culture required to effect in vitro maturation must therefore be considered a potential risk and a possible cause of the poor developmental competence usually associated with IVM. In its current state, human IVM is at an early stage of development and for clinical use, must be considered experimental and not without the potential for unanticipated downstream consequences.

CLINICAL PREGNANCIES RESULTING FROM HUMAN IVM

The full developmental competence of an oocyte and an embryo is ultimately determined only by live births and may arguably also require close follow-up after birth to ensure normal development. Veeck et al (1983) reported the first live birth resulting from successful IVM as part of an IVF program in which patients received ovarian stimulation drugs. Subsequently, Cha et al (1991) reported IVM with unstimulated oocytes from ovariectomies in a donor oocyte programme. Following these reports, a number of studies described the successful use of IVM resulting in clinical pregnancies (Trounson et al., 1994; Barnes et al., 1995; Nagy et al., 1996; Edirisinghe et al., 1997; Jaroudi et al., 1997, 1999; Liu et al., 1997; Cha and Chian, 1998; Russell, 1998; Thornton et al., 1998; Tucker et al., 1998; De Vos et al., 1999; Chian et al., 1999a,b, 2000, 2001; Mikkelsen et al., 1999, 2000; Cha et al., 2000; Smith et al., 2000; Abdul-Jalil et al., 2001; Wu et al., 2001). However, it is important to emphasize that live birth rates with IVM remain disappointingly low (usually less than 10%) and variable.

ROLE OF PRIOR OVARIAN STIMULATION IN HUMAN IVM

To achieve ovarian stimulation for routine IVF, gonadotrophin-releasing hormone (GnRH) superagonists are used to suppress the secretion and release of endogenous gonadotrophins from the pituitary gland. Exogenous FSH or FSH + LH is administered to induce multiple follicular growth and the final phase of oocyte maturation is induced by a dose of hCG to emulate the LH surge.

Various protocols including partial or minimal exogenous hormonal stimulation are used in preparation for IVM. In preliminary studies, the treatment of women for one or three days with recombinant human FSH (rFSH) early in the follicular phase showed no difference in the recovery rate of oocytes, or frequency of maturation, fertilization or embryo development in culture (Trounson et al, 1998). Similar results were reported by Mikkelsen et al (1999) who found that after the treatment of with rFSH for three days, on days 3-5 of the cycle, extending the rFSH pre-treatment from three to six days to produce follicles >10mm in diameter had no benefit. However, Wynn et al

(1998) administered a truncated course of 600IU rFSH over five days (300IU on day two, 150IU on days four and six). They reported an increase in oocyte recovery to a mean of 7.5 oocytes after rFSH treatment compared with 5.2 oocytes from untreated patients. After 48 hours in culture, supplemented with FSH and hCG, significantly more oocytes completed maturation to MII following FSH stimulation than in untreated women (71.1% and 43.5% respectively) and significantly fewer degenerating oocytes were seen both at the time of collection and after 48 hours of culture. It is possible that such in vivo stimulation encouraged the production of LH receptors that were stimulated by hCG during IVM. However, evidence to support this notion is lacking.

Suikkari et al (2000) also investigated the use of minimal ovarian stimulation. In a natural menstrual cycle, because serum FSH begins to increase in the late luteal phase, a low dose rFSH (37.5 IU/day) was administered starting in the late luteal phase (11 days post LH surge) in two groups of women, those with regular menstrual cycles and women with polycystic ovarian syndrome (PCO) and irregular cycles. In women with anovulatory cycles, a withdrawal bleed was induced and low-dose rFSH commenced on the ninth of ten days of progesterone administration. Gonadotrophin administration was continued until the leading follicle was approximately 10mm diameter. Oocytes were retrieved after withdrawing rFSH for 2-5 days. It was postulated that low dose rFSH priming of follicles would support the growth of multiple follicles, thereby increasing the number of immature oocytes retrieved for IVM. A good yield of immature oocytes (11.2 and 11.5: regular and irregular menstrual cycles, respectively) was obtained for both groups of women, 71% of immature oocytes reached MII after 44 hours in culture, and 64% of these fertilized after ICSI. These results did not differ significantly between the two groups of women.

The time course of GVBD and maturation to MII differ between GV oocytes retrieved from stimulated and unstimulated ovaries. Cha and Chian (1998) showed that when compared oocytes obtained from unstimulated ovaries, GVBD occurs significantly earlier in oocytes obtained from stimulated ovaries. After 12 hours of culture, 80% of GV stage oocytes from stimulated ovaries had undergone GVBD, whereas those from unstimulated ovaries remained unchanged. As a result, the completion times for nuclear maturation, shown by extrusion of the first polar body differed; with ~75% of oocytes from stimulated ovaries at MII by 30 hours of culture, whereas 75% of oocytes from unstimulated ovaries reached MII by 42-45 hours (Cha and Chian, 1998; Trounson et al, 2001). However, the final proportion of maturing oocytes derived from stimulated and unstimulated ovaries was not different. Exposure of the stimulated follicles to FSH and/or hCG may account for the difference in timing of GVBD, potentially due to altered receptor profiles or post-receptor signalling sensitivities, although the precise mechanisms remain to be elucidated.

Gonadotrophin therapy leads to the growth of multiple follicles and is associated with elevated estradiol concentrations. Consequently, oocytes retrieved from stimulated ovaries have been subject to a predominantly estrogenic milieu (Cobo et al., 1999). Furthermore, the addition of estradiol to oocyte maturation medium may directly influence the quality of the maturing oocyte. Whilst no apparent effect of estradiol on either GVBD or further progression of meiosis was detected, increased fertilization and cleavage rates were observed after IVM. Estradiol induces a series of transient increases in the intracellular free Ca^{2+} concentration, which may contribute to the oocyte's competence for fertilization and early post-fertilization development (Tesarik and Mendoza, 1995).

One small study has suggested that growth hormone administration during ovarian stimulation for ICSI may promote maturation in vitro and fertilization (Hassan et al., 2001), however, an unusually high number of immature oocytes was collected (1-2 per patient), and some differences were found between the patient groups with and without growth hormone in terms of ICSI outcome. While this finding suggests that the results may not be specific to maturing oocytes, the results remain to be confirmed.

IVM and fertilization have also been reported for human immature oocytes retrieved at the germinal vesicle stage after the administration of exogenous hCG in vivo (Veeck et al., 1983; Prins et al., 1987; Dandekar et al., 1991; Toth et al., 1994; Janssenswillen et al., 1995; Nagy et al., 1996; Edirisinghe et al., 1997; Farhi et al., 1997; Goud et al., 1998; Thornton et al., 1998; Tucker et al., 1998; De Vos et al., 1999; Chian et al., 1999a,b; 2000; Cavilla et al., 2001; Abdul-Jalil et al., 2001). These studies differ in many respects, e.g. presence or absence of prior FSH stimulation, number of immature oocytes for in vitro maturation, time in culture, culture medium used and maturation, fertilization and embryo cleavage rates. Immature oocytes retrieved after the in vivo administration of hCG can be matured in culture without gonadotrophin or steroid supplement (Veeck et al., 1983; Dandekar et al., 1991), but the culture medium usually contains the patient's own serum and occasionally granulosa cells recovered from the same follicle or from follicles that contained a mature follicle. It is possible that hCG may have initiated the process of maturation, even if oocytes are still in the GV stage when collected.

CRYOPRESERVATION OF IMMATURE OOCYTES

Collection of oocytes earlier than their normal time of ovulation shortens not only the oocyte growth phase, but also the proliferative phase of endometrial development, potentially causing difficulties in ensuring endometrial receptivity in the same cycle. Cryopreservation of immature oocytes would facilitate replacement of embryos in a subsequent natural or hormone replacement cycle. Toth et al. (1994) demonstrated that GV stage

oocytes from stimulated IVF cycles can survive cryopreservation and resume meiosis to achieve full nuclear maturation, retaining the same capacity for fertilization and development as control (cultured, non-cryopreserved immature) oocytes. Tucker et al. (1998) reported a pregnancy from cryopreserved GV oocytes. A total of 29 oocytes were cryopreserved, 16 MII and 13 GV, but only three GV oocytes survived. After 30 hours of culture, two oocytes had matured, which fertilized after ICSI. Tucker et al. (1998) concluded that this study proves the feasibility if not the efficiency of coupling cryopreservation and IVM (for additional details and discussion of oocyte cryopreservation, see Picton, this volume).

THE SPECIAL CASE OF POLYCYSTIC OVARIES (PCO)

Polycystic ovaries (PCO) occur when antral follicles of ~3-8mm accumulate in the cortex and remain under an androgen-dominated environment due to increased thecal cell secretion of androgens and a blockage of aromatization in the granulosa compartments (Almahbobi and Trounson, 1996). Dominance of a particular follicle may fail to occur. These peripheral antral follicles are more accessible and greater in number than in a non-polycystic ovary.

In 1994, Trounson et al. developed methods for the recovery of immature oocytes from the ovaries of patients with PCO. However, Barnes et al. (1996) demonstrated significantly reduced maturation, fertilization and embryo development for oocytes from women with polycystic ovary syndrome (PCOS), compared with those recovered from regularly cycling women without PCOS. They suggested that the abnormal endocrine environment and stasis of follicular growth in PCOS patients may disrupt the oocyte, although incipient follicular atresia does not appear to reduce the developmental competence of human oocytes (Barnes et al., 1996). Despite the potentially reduced competence of oocytes, blastocysts have been produced and live births have occurred as a result of IVM of oocytes from women with PCOS (Trounson et al., 1994; Barnes et al., 1995; Cha and Chian, 1998; Chian et al., 1999a,b, 2001). Furthermore, the higher oocyte yield from the increased numbers of antral follicles in such patients may potentially compensate for lower individual viability of oocytes (Trounson et al., 1996; Child et al., 2001). Oocyte yield is directly correlated with the number of antral follicles visible by ultrasound in the early follicular phase and a good oocyte yield is associated with a higher chance of pregnancy (Tan et al., 2002). When performing an immature oocyte recovery in a patient with PCO and irregular menstrual cycles, inducing a withdrawal bleed may be advisable, causing the endometrium to develop afresh and a new cohort of antral follicles that have not arrested their growth to be recruited.

Chian et al (1999a,b) reported a clinical pregnancy rate of 39% per cycle in women diagnosed with polycystic ovaries who were primed with hCG prior

to aspiration of immature oocytes for IVM. The patients had irregular menstrual cycles and withdrawal bleeding was induced by the administration of intravaginal progesterone for 10 days with hCG administered 10-14 days after steroid withdrawal. Transvaginal oocyte recovery from antral follicles was performed, and oocytes were matured for 24-48 hr in TCM 199 containing 20% maternal serum, 25mM pyruvic acid and 75mIU/ml hMG. These authors claimed that priming with hCG prior to oocyte recovery increased the developmental competence of the immature oocytes collected. However Trounson et al. (2001) argued that no data were provided to show that the oocytes had remained immature after hCG injection. Furthermore, Chian et al. (2000) had shown that 46% of oocytes were already maturing in hCG-primed patients, and they completed meiosis 12-24 hours earlier than oocytes from unprimed patients. Trounson et al. (2001) found no significant difference between final fertilization, embryo development and pregnancy rates for hCG primed and unprimed patients.

The application of human oocyte maturation in vitro is a potentially suitable adjunct to surgery or diathermy of ovaries for the establishment of pregnancy in infertile women with PCOS; however, the success which can be expected varies considerably according to published reports and in different centers. It is clear that improvements must be made to the maturation conditions to retain the potential developmental competence of immature oocytes and this should be the priority for research on oocyte maturation.

FERTILIZATION OF IVM OOCYTES

There is evidence that removing human oocytes from their normal follicular environment before its luteinization yields oocytes with compromised ability to be fertilized, as exemplified by the failure to elicit normal calcium signalling in response to sperm-oocyte fusion (Herbert et al., 1997). ICSI of in vitro matured oocytes increases the likelihood of normal fertilization; overcoming such problems as zona hardening due to extended culture (DeFelici and Siracusa, 1982; Barnes et al., 1995, 1996; Nagy et al., 1996; Cha and Chian, 1998; Hwang, et al., 2000) and failure of the cortical reaction, likely due to the inadequate peripheral migration of cortical granules during maturation. Consequently, intracytoplasmic sperm injection (ICSI) is now employed almost universally to achieve fertilization in human IVM programs. Nevertheless, since failure of cortical granule orientation may be considered evidence of ooplasmic immaturity or abnormality, we should be open to the possibility of other cytoplasmic anomalies originating or being exacerbated by IVM that may potentially effect outcome or the resulting offspring.

IN VITRO STIMULATION OF OOCYTE MATURATION

The content of the medium used for IVM may effect the outcome of maturation and subsequent development. A key experiment in the 1990s, which re-awakened interest in human IVM, used human follicular fluid to supplement the culture medium (Cha et al., 1991) but, with the growing preference for defined media, the roles of individual factors are now being assessed and the inclusion of biological fluids is declining.

Various hormones and growth factors included in the culture medium, such as epidermal growth factor (EGF, Gómez et al., 1993a,b; Goud et al., 1998) or follicle stimulating hormone (FSH, Barnes et al., 1996; Durinzi et al., 1997) with or without human chorionic gonadotrophin (hCG, Jaroudi et al., 1997; Liu et al., 1997; Cha et al., 2000) may promote oocyte maturation and subsequent embryo development. Other substances, having positive effects on maturation and fertilization in other species that have not been tested in humans to date include glutathione and cysteine (Jeong and Yang, 2001), and it is noteworthy that the actions of most currently are believed to be on granulosa cells, rather than directly on the oocyte. In any event, the granulosa cells may modify and metabolize components of culture medium, thereby conditioning the medium, and effecting the oocyte either directly via modified communication at gap junctions, indirectly via paracrine effects, or physically, by modification of their presence and orientation around the oocyte (see Albertini, this volume). IVM is most successful when at retrieval; the oocyte is closely surrounded by unexpanded cumulus cells that remain in the culture until maturation is complete. Cumulus cells are highly metabolically active and their secretions, such as steroids (see Antczak, this volume), may be influenced by hormonal supplementation in vitro (Durinzi et al., 1997). While oocyte maturation is believed to occur principally by the alleviation of inhibitory influences from granulosa cells, including those passed to the oocyte via gap junctions, local stimulatory factors may augment the process.

GONADOTROPHINS

Gonadotrophins are the major ‘instigators’ of oocyte maturation *in vivo*. Therefore, they are prime candidates as supplements intended to promote maturation *in vitro*. However, the actions of gonadotrophins at the follicular level are more complex than might be anticipated from their widespread and predictable application as systemic inducers of ovulation.

Granulosa cells contain mRNA for FSH receptors from very early in follicle development (Oktay et al., 1997), whereas LH receptors are induced in response to FSH stimulation, and are principally located at the periphery of large follicles (Amsterdam et al., 1975). As follicles grow, major variations arise between granulosa cells in different parts of the follicle (Gersak et al,

1996; Rodgers et al, 2001), for example, the inner, cumulus cells continue growing and respond to FSH by mucification, whilst the outer mural granulosa cells express P₄₅₀ aromatase and LH receptors (Gosden et al. 1993). Cumulus cells present in IVM cultures have few LH receptors. Interestingly, exposure of mouse oocyte cumulus complexes to FSH in vitro may result in inappropriate maturation associated with precocious and abnormal LH receptor expression (Eppig et al, 2000). Moreover, different isoforms of FSH may vary in their effects, and the possible consequences of selecting one isoform over another for IVM are unclear (Yding Andersen et al, 1999; Vitt et al, 2001).

Spontaneous meiotic maturation and FSH-induced maturation are mediated by different intracellular pathways as demonstrated by their differing sensitivity to calcium chelation (Coticchio and Flemming, 1998). Similarly, it has been suggested that FSH-induced maturation be mediated by the generation of a stimulatory signal, which overcomes an inhibitory input from the follicle. However; spontaneous maturation does not require a stimulatory signal as it is believed to result from the removal of follicular inhibitors only (Downs et al., 1993). For clinical IVM, FSH and LH or hCG are usually added to cultures in an attempt to mimic *in vivo* surge, and these hormones are considered relatively 'safe' because of prior patient exposure. While dose-response data in humans is lacking, in rhesus monkeys, the addition of gonadotrophins *in vitro* significantly promoted fertilization and embryo development (Morgan et al, 1991). In macaques, FSH combined with LH for *in vitro* maturation stimulated faster post-fertilization development (Weston et al, 1996).

EPIDERMAL GROWTH FACTOR (EGF)

EGF was first described in 1962 and was after isolation from mouse submaxillary glands (Cohen, 1962). Human EGF, initially called urogastrone, was subsequently isolated from human urine (Starkey et al., 1975; Cohen and Carpenter, 1975) and is a member of the peptide growth factor family (Cooke et al., 1987). As a single polypeptide chain of 53 amino-acids, it is relatively small (~6 kDa) and of a size that is permissive for easy penetration through the zona pellucida of the human oocyte with the potential to exert its effect directly on the oocyte (Shalgi et al, 1973; Goud et al., 1998).

EGF has mitogenic effects in a variety of mesodermal and ectodermal tissues and is involved in regulating cell proliferation (Reeka et al., 1998) and in the ovary, mRNA for prepro-EGF has been identified in primordial follicles of newborn mice (Rall et al., 1985). EGF is synthesised in small human antral follicles (Westergaard et al., 1990) and may have a role in follicular growth, where rapid cell proliferation occurs (Das et al., 1991; Lonergan et al., 1996; Goud et al., 1998; Qu et al., 2000). TGF- α , which also uses the EGF receptor, is a potent angiogenic agent (Yeh et al., 1993),

modulates granulosa cell steroidogenesis and participates in follicular maturation (Reeka et al., 1998).

The EGF receptor is present on many cells of the ovary, including the oocytes of human primordial (Qu et al., 2000), primary and pre-antral follicles (Maruo et al., 1993; Tamura et al., 1995), as well as granulosa cells of antral follicles (Reeka et al., 1998). EGF may play a positive role in oocyte maturation in vitro (Gomez et al., 1993a; Singh et al., 1997; Goud et al., 1998), and it may also augment maturation and development of intrafollicular oocytes in follicle culture (Boland and Gosden, 1994; Smitz et al., 1998). A greater understanding of the roles of EGF in the development of the follicle and oocyte may be an important prerequisite in the development of clinically effective in vitro growth and maturation systems.

MEIOSIS ACTIVATING STEROL (MAS)

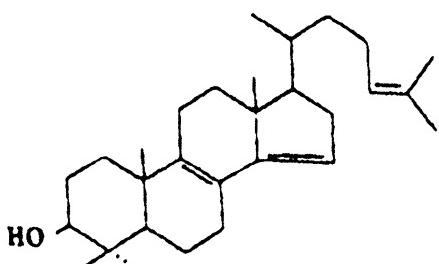
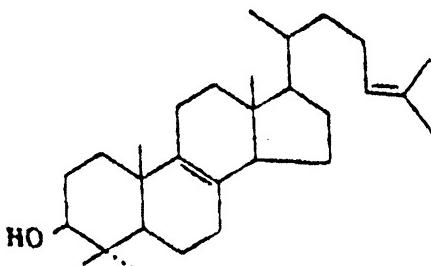
Byskov et al. (1995) discovered a substance which they termed meiosis-activating sterol (MAS) in view of its ability to initiate meiosis in germ cells of foetal mouse testes, and to induce resumption of meiosis in vitro of hypoxanthine-arrested mouse oocytes (Byskov et al., 1998). Two naturally occurring sterols which induce meiotic resumption of both naked and cumulus-enclosed mouse oocytes in vitro have been identified: FF-MAS, extracted from human preovulatory follicular fluid, and T-MAS extracted from bull testicular tissue (Byskov et al., 1998). The chemical structures of these sterols are shown in figure 10.5.

FF-MAS and T-MAS are intermediates in the cholesterol biosynthetic pathway, however, MAS accumulates only in the gonads (Byskov et al., 1997, 1998). FSH and hCG directly effect cholesterol biosynthesis (Douglas et al., 1978; Baranao and Hammond, 1986; Leonardsen et al., 2000). Within the preovulatory follicle, timely resumption of oocyte meiosis has been hypothesised to arise from the combined effects of altered sterol biosynthesis by the granulosa and cumulus cells, resulting in the accumulation of MAS, and direct transfer of MAS from the cumulus cells to the oocyte via gap junctions (Leonardsen et al., 2000). Hence MAS may be an endogenous mediator of gonadotrophin action on oocytes.

Several studies have tested the effectiveness of purified or synthetic FF-MAS in stimulating maturation in vitro. However, stimulation of endogenous production may be more effective than supplementation, because exogenous MAS is probably readily converted to cholesterol and other steroids (Leonardsen et al., 2000). Pharmacological inhibition of $\Delta 14$ -reductase in hypoxanthine-arrested mouse cumulus-oocyte complexes caused accumulation of endogenous FF-MAS and resumption of oocyte meiosis (Leonardsen et al. 2000). The same effect was not observed in naked oocytes, leading Leonardsen et al. (2000) to propose that MAS biosynthesis occurs in

the cumulus cells and not in the oocyte. Blocking downstream metabolism of FF-MAS also stimulated GVBD in cumulus-enclosed but not denuded

FF-MAS



T-MAS

FIGURE 10.5. Structural diagrams of FF-MAS and T-MAS.

oocytes. Downs et al. (2001) proposed an alternative interpretation, that induction of meiotic maturation by inhibitors of enzymes situated downstream of FF-MAS in sterol biosynthesis may be acting by preventing accumulation of inhibitory downstream metabolites such as cholesterol. This is a plausible explanation since the inhibitor used has an additional site of action at $\Delta 7$ -reductase that produces cholesterol from 7-dehydrocholesterol. Indeed, cholesterol was shown to augment the inhibitory effect of dbcAMP in a dose-dependent manner in both cumulus-enclosed and denuded oocytes.

Ketoconazole inhibits the cytochrome P450-14DM enzyme, which converts lanosterol to FF-MAS in vitro (Yoshida et al., 1996). In 1998, Tsafiriri et al. reported no effect of ketoconazole (a potent inhibitor of sterol synthesis) on oocyte maturation in the rat, both in vivo gonadotrophin-stimulated oocytes and spontaneously maturing oocytes in vitro. However,

stimulation of P450-14DM by gonadotrophins opposes an inhibitory effect by ketoconazole on this enzyme in the living animal (Byskov et al., 1999), so the balance of enzyme activity locally in the cumulus is not readily quantifiable.

The signalling pathways for FF-MAS during resumption of oocyte meiosis are unknown. Protein synthesis is essential to maintain the oocyte's responsiveness to MAS, however; transcription is not required (Grondahl et al, 2000). The MAS receptor has not been identified, although Faerge et al. (2001) found specific binding of FF-MAS to predominate at the oolemma of denuded oocytes. Janowski et al (1996) observed FF-MAS to be an activating ligand for the orphan nuclear receptor LXR- α , in common with other sterols, but Grondahl et al (1998) found that none of the other ligands, or cholesterol, induced resumption of meiosis in hypoxanthine-arrested mouse oocytes.

It is clear that the control of oocyte maturation at the molecular level remains to be elucidated and many other locally active factors will have a role. The follicular milieu is a rich and complex mixture of hormones, growth factors, macromolecules and binding proteins, and represents an environment honed by evolution for the primary purpose of viable oocyte production. It is therefore not surprising that the addition of a few selected factors to culture media does not overcome the loss of competence or the inability to acquire competence in vitro. However, intense and focused efforts are essential if IVM is ever to realize its widely anticipated clinical promise as discussed below.

POTENTIAL BENEFITS OF IVM

One anticipated benefit of IVM is that it could eventually be an alternative or effective offer an alternative or an adjunct to the current ovarian stimulation protocols used in IVF. For example, IVM might allow the 10-15% of immature oocytes recovered after ovarian stimulation to be rescued, reducing wastage of oocytes (Cha and Chian, 1998). This would particularly benefit patients with an unsynchronized cohort of follicles who have a high proportion of immature oocytes collected after stimulation. While some of these may mature spontaneously in vitro as a result of removal from the follicles, which is more likely if the cumulus remains intact, support with an appropriate in vitro environment that may include gonadotrophin or other supplements may be beneficial for success rates and outcome.

IVM would reduce the need for gonadotrophin stimulation, which may even become redundant, greatly reducing the cost of IVF treatment. Further savings would be contributed by reduced ultrasound and biochemical monitoring in the absence of gonadotrophin stimulation. This can be predicted because research has shown that immature oocytes collected from follicles around 5-to-14mm diameter can be used to achieve pregnancies, albeit not at optimal efficiency at present. These follicles may be aspirated during unstimulated or minimally stimulated cycles, including follicles from

polycystic ovaries (see below). The main criteria in choosing the time for oocyte retrieval are that the follicle has not undergone more than the initial stages of atresia, (i.e. follicle dominance is not firmly established), and that the follicle(s) should be sufficiently large for the corresponding oocyte(s) to have achieved the threshold for developmental competence. The ideal time and environment for such oocytes to be collected remains to be established.

IVM might particularly benefit patients having concurrent PCO and infertility. Such patients may over-respond to gonadotrophin stimulation for IVF, potentially resulting in ovarian hyperstimulation syndrome (OHSS; Rizk and Smitz, 1992; MacDougall et al., 1993). While mild OHSS is of little clinical relevance (Rizk and Aboulghar, 1999), severe OHSS characterised by massive ovarian enlargement and fluid accumulation in the abdominal and pleural cavities (third spacing) often is coincident with gross changes in blood chemistry and is a life threatening complication with a risk of thrombosis and death (Cluroe and Synek, 1995). The risk of OHSS could be minimised and hence drug-free IVM treatment may be associated with reduced health risks for the mother.

In contrast to mature oocytes, immature oocytes in antral follicles at the late follicular phase can be obtained more readily from patients undergoing Caesarean section or oophorectomy, if these interventions are carefully timed (Hwu et al., 1998; Cha and Chian, 1998). The increased supply of mature oocytes derived by IVM would have a number of uses in subsequent treatment or research. For the future, a combination of immature oocyte cryopreservation, IVM and IVF could provide the opportunity to establish oocyte banks.

IVM might provide a chance of fertility preservation in women with cancer or other malignant conditions who undergo normal IVF cycle prior to sterilizing chemotherapy, or in whom ovarian stimulation is contraindicated, particularly if their cycle timing is appropriate for immature follicle aspiration. Such an application of IVM might particularly benefit those women for whom IVF for embryo cryopreservation is inappropriate or unacceptable. Immature oocyte cryopreservation for subsequent IVM also has the potential to avoid many of the legal and ethical problems associated with embryo cryopreservation. As a research tool, IVM may provide a valuable model for investigating the causes of meiotic aberrations and aneuploidies, and other anomalies which are common in mature human oocytes (Gras et al., 1992; Delhanty et al., 1997). However, for all of these sanguine notions to reach fulfilment and before IVM can be considered as a routine treatment for human infertility, validated and efficient systems of maturation, fertilization and embryo development are required that produce acceptable levels of confidence regarding safety. While there are potential risks attached to the prolonged culture associated with IVM, it is important to note that reports to date on human IVM offspring have not given cause for alarm. Therefore, despite present the limitations of IVM, we suggest that the potential benefits

of IVM compared to conventional IVF, together with the developing knowledge on mechanisms controlling follicle and oocyte growth and early embryology may provide an opportune time to begin to a serious consideration of IVM with the caveat that it be applied with caution.

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CHAPTER ELEVEN

WHAT IS THE ROLE OF MITOCHONDRIA IN EMBRYO COMPETENCY?

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INTRODUCTION

The role of mitochondria in the establishment of developmental competence for the preimplantation mammalian embryo is an area of growing interest for researchers, embryologists and clinicians involved in in vitro fertilization (IVF). This review describes some of the more clinically significant issues in IVF that may be associated with the function and regulation of mitochondria during the earliest stages of embryonic development. In somatic cells, mitochondria have a central role in metabolism, cellular aging and apoptotic cell death, and specific cytopathologies may originate from the accumulation of *de novo* mitochondrial genetic defects that can effect normal cell and tissue function. Maternally inherited mitochondrial DNA (mtDNA) defects can result in debilitating or lethal conditions for affected individuals (so-called mitochondrial genetic diseases) if genes coding for respiratory chain enzymes are involved (oxidative phosphorylation diseases, OXPHOS). For the oocyte and early embryo, it has been proposed that mutations in the mitochondrial genome may contribute to impaired function and developmental incompetence (Chen et al., 1995; Keefe et al., 1995; Brenner et al., 1998; Barritt et al., 1999,2000). The extent to which specific mtDNA defects are related to early reproductive failure is not clear, but genetic mutations may result in diminished ATP content leading to embryonic abnormalities such as cleavage arrest, slow development, blastomere loss by apoptosis and ultimately, implantation failure (Van Blerkom et al., 1995; Van Blerkom et al., 2000,2001). As described below, the mitochondrial complement in human oocytes can be in excess of 150,000 organelles, so any potential adverse effect of mtDNA mutations associated with respiratory function would depend upon

the magnitude of the mutant population (mutant load). It has also been suggested that embryonic failures could be a consequence of asymmetrical mitochondrial distribution in the pronuclear embryo. This could lead to disproportionate mitochondrial inheritance and perhaps blastomeres with diminished ATP generating capacity which could reduce developmental competency (Van Blerkom et al., 1995; Van Blerkom et al., 2000). Although it is generally assumed that mitochondria are critical determinants of competence, the extent to which differences in early human embryo development observed within and between cohorts is associated with mitochondrial distribution and metabolic capabilities is unknown.

It has long been thought that the transmission of mitochondria between generations is strictly maternal. However, the development of more invasive techniques of assisted reproduction has increased the concern that the mechanisms of uni-parental maternal mitochondria inheritance may be altered and as a result, could comprise the genetic integrity of the offspring. For example, there is evidence for the persistence of human paternal mtDNA in "abnormal" unfertilized oocytes and embryos (St. John, et al., 2000a; St. John et al., 2000b; St. John et al., 2001). Whether human sperm mtDNA is entirely eliminated or whether certain embryos are incapable of destroying sperm mitochondria is unknown, and it is unclear whether the detection of paternal mtDNA sequences at extremely low levels is of any biological significance or simply coincidental and unrelated to embryo competence. With the current experimental efforts designed to evaluate the potential therapeutic applications of nuclear and cytoplasmic transplantation for curing inheritable mitochondrial disease or known chromosomal abnormalities, it is necessary to understand the role(s) of mitochondria and the mechanisms that regulate their function in early mammalian embryogenesis. An understanding of the regulation and function of mitochondria in early human development becomes especially relevant in clinical IVF owing to recent reports of three children born after inter-ooplasmic transfer in which a significant expansion of donor mtDNA has occurred and resulted in two genetically distinct populations of mtDNA coexisting within the same cytoplasm, a condition known as heteroplasmy (Barritt et al., 2000, 2001; Brenner et al., 2000).

DOES MITOCHONDRIAL DNA CONTENT EFFECT OOCYTE AND EMBRYO COMPETENCE?

Mitochondria are maternally inherited organelles that provide levels of ATP via oxidative phosphorylation necessary for normal biosynthetic, metabolic and physiological process. Oocytes have greatly enriched numbers of mitochondria that can support essential developmental processes such as oocyte growth and meiotic maturation in addition to providing the respiratory

engine for early embryonic development. However, mammalian embryos are unique in that mitochondrial replication does not occur until gastrulation. In the pre-migratory germ cells there may be as few as 10 mitochondria which increase to around 200 in each oogonium. At menarche, each of the approximately 300,000 resting primordial oocytes that have survived previous waves of atresia contains about 6000 mitochondria. The fully-grown, meiotically mature (metaphase II, MII) human oocyte contains between 100,000-800,000 mitochondria, a remarkable range when it is considered that each organelle probably contains a single mtDNA copy (Chen et al, 1995; Jansen and deBoer, 1998; Barritt et al, 2001; Reynier et al, 2001). In the absence of detectable mitochondrial replication during the preimplantation stages, the prior expansion of these organelles during oogenesis presumably is sufficient to support levels of oxidative phosphorylation required for normal function until replication resumes (Cummins 1998; Piko and Taylor 1987).

Early studies by Piko and Matsumoto (1976) estimated by transmission electron microscopic morphometric analysis that the mitochondrial content of fully-grown mouse oocytes was approximately 92,500 copies/oocyte. Michaels (1982) used DNA hybridization on pooled bovine oocytes to derive an average complement of 260,000 mtDNA copies/oocyte. In 1987, Piko and Taylor used dot blot analysis in pooled mouse oocytes to estimate an average mtDNA copy number of 119,000. With a more accurate competitive PCR method, Chen (1995) reported that the average number of mtDNA copies in a small cohort of human oocytes was 138,000. De Boer (1999) used electron microscopy and volumetric multiplication of the number of mitochondria counted in representative thin sections to determine mitochondrial numbers in human oocytes and reported that the average oocyte may contain as many as 400,000 organelles. However, the accuracy of this estimate is questionable because the distribution of mitochondria may not be uniform.

Given the wide ranges of mitochondrial DNA numerical estimates in oocytes, studies using a sensitive, fluorescence-based, real-time, rapid cycle PCR amplification technique have been used to quantify mtDNA in single during real-time PCR. In a preliminary study, Steuerwald et al (1999) estimated that the average mtDNA copy number/oocyte was approximately 314,000, a figure relatively close to the one reported by de Boer (1999). In a subsequent study that attempted to correlate mtDNA copy number and the degree of genetic variation within the mitochondrial complement that could be related to reproductive senescence, Barrit et al (2002) examined 87 MII human oocytes from 29 patients and detected an average mtDNA copy number of 795,000 ($\pm 243,000$). Similar to the findings of Reynier et al (2001), mitochondrial DNA content was found to vary considerably between oocytes in the same and different cohorts, but no significant relationship between mtDNA copy number and maternal age was identified. While an

exponential increase in mtDNA deletions in postmitotic cells of brain and muscle tissue is characteristic of advanced chronological age (Kadenbach et al., 1995), a similar increase does not appear to effect the oocytes of women of advanced reproductive age.

Whether a relationship exists between mtDNA content and fertilizability has also been investigated. Rainier et al (2001) used real-time quantitative PCR to analyze 113 unfertilized oocytes obtained from 43 patients and reported an average mtDNA content of 193,000 copies/oocyte with an oocyte-specific range between 20,000- to- 598,000. These investigators reported that the average mitochondrial DNA copy number was significantly lower in cohorts suffering from fertilization failure as compared with cohorts with normal rates of fertilization. Reynier et al (2001) proposed that mtDNA content is associated with both nuclear and cytoplasmic maturational competence of the oocyte with some proportion of IVF failures related to inadequate mitochondrial biogenesis. This hypothesis needs further confirmation and a non-human primate model may be appropriate. While it is assumed that a one-to-one relationship exists between mtDNA copies and mitochondrial numbers, such a correspondence should be evident by morphometric analysis, especially if such extremes (20,000-to-800,000) actually exist. This nature of the relationship between the size of the mitochondrial complement and mtDNA copy number also requires additional study.

In a recent study, Barrit et al. (2002) examined the mtDNA content in individual germinal vesicle (GV) and MII oocytes and reported no significant differences, which indicates that mtDNA replication is completed by the GV stage. Confirmation of this finding may require the challenging task of analyzing the same oocyte during meiotic maturation, assuming that non-invasive microscopic techniques could be developed with adequate sensitivity to quantify mitochondria. Large variations in oocyte-specific ATP content have been reported and the level of ATP production may be associated with the developmental competence of the embryo (Van Blerkom et al., 1995, 1998, 2000). However, it is difficult to determine whether a significant relationship exists between embryo competence, mitochondrial content and function since most studies require the destruction of human gametes and embryos for analysis. Whether mtDNA copy number correlates with oxidative metabolism and ATP generation needs further investigation. However, perhaps the absolute number of mitochondria in an oocyte is not as developmentally relevant as the proportion of functional mitochondria engaged in ATP production or during cleavage, the uniformity of their distribution between blastomeres. In this respect, the notion that all mitochondria present in a human oocyte are active in respiration is an untested assumption, and may be unwarranted owing to the fact that oocytes

with high mutant loads of mitochondria with known respiratory deficiencies are nevertheless fertilizable and competent. Because mitochondria do not resume replication until well after implantation, they must be inherited by all resulting blastomeres and cells of the preimplantation embryo in order for development to progress normally. Perhaps a minimum or baseline level of functionally active organelles is all that is required to sustain the resumption of meiosis, fertilization, preimplantation and early fetal development. If so, then the possibility of differential mitochondrial distribution and functionality in human oocytes and embryos could have important clinical implications in understanding the variable competence exhibited by normal appearing embryos within cohorts.

DO MITOCHONDRIAL DNA MUTATIONS EFFECT OOCYTE AND EMBRYO FUNCTION?

There are now more than 150 known mtDNA rearrangements detected in somatic cells, including deletions, insertions and duplications (Wallace, 1993). These mutations are responsible for a number of catastrophic neuromuscular diseases, such as Kearns-Sayre syndrome (KSS), chronic progressive external ophthalmoplegia (CPEO), and Pearson's syndrome. Mitochondrial DNA rearrangements have been shown to accumulate with age, and become predominant in post-mitotic, non-dividing tissues (Cortopassi and Arnheim, 1990). Despite the fact that glycolysis is a very significant source of ATP during the latter preimplantation and early peri-implantation stages, it is generally assumed that there needs to be a sufficient number of normal mitochondrial genomes in order for the embryo to develop and implant. Although a relationship between mitochondrial mutations and reproductive success is unproven, it seems reasonable to suggest that a high frequency of genetically abnormal mitochondria in the oocyte could reduce the number of functional mitochondria leading to embryonic arrest, failed implantation, or mitochondrial disease. However, embryonic arrest would be expected to involve mutations that eliminate most or all respiratory activity in a sizeable population of mitochondria because children are born with inherited mtDNA defects that reduce metabolic efficiency and which presumably occur at high load in the oocyte.

Several mutations of mitochondrial DNA have been associated with defined clinical syndromes arise as sporadic large-scale rearrangements (deletions and duplications) or maternally inherited point mutations. The clinical manifestations of mitochondrial mutations are extremely heterogeneous, ranging from myopathies, encephalomyopathies and cardiopathies to complex multisystem syndromes. The point mutations of mtDNA can occur in mRNA, tRNA or rRNA of the mitochondrial genome.

Also, there is a correlation between the severity of the clinical and biochemical phenotype and the degree of mtDNA heteroplasmy in the somatic tissues. The most common clinical syndrome associated with point mutations of mtDNA is MERRF, where there is a single base pair transition of A to G at position 8344 of the mitochondrial genome in the tRNA^{lys} gene. MELAS, another common disease characterized by a heteroplasmic point mutation in another tRNA^{lys} gene has a nucleotide transition of A to G at base pair position 3243. Large rearrangements in mitochondrial DNA (mtDNA) in somatic cells are responsible for certain neuromuscular syndromes, the most common being Kearns-Sayre syndrome (KSS) or progressive external ophthalmoplegia (PEO).

Multiple laboratories have detected a particular mtDNA mutation called the "common deletion," mtDNA4977, in human oocytes (Chen et al., 1995; Keefe et al., 1995; Brenner et al., 1998; Barritt et al., 1999). The mtDNA4977 mutation can be detected at a frequency of 30% to 50% in human oocytes but the most important issues are whether the frequency of mutation increases with maternal age and whether frequency corresponds to oocyte quality and embryo developmental competence. It is now generally thought that no age-related increase in the frequency of this mutation occurs and only one report, a study by Keefe (1995), showed a significant association with maternal age. There is some evidence that a significant reduction in the frequency of mtDNA 4977 occurs in embryos when compared to oocytes in the same cohort (Brenner et al., 1998; Barritt et al., 1999). If confirmed, this finding could suggest that a selection mechanism might occur in the oocyte or early preimplantation embryo to reduce or eliminate the inheritance of mitochondrial mutations, perhaps by apoptosis. The biological significance, if any, of this mutation needs to be determined. Additionally, 23 novel mtDNA rearrangements have been identified in human oocytes and embryos (Barritt et al., 1999). Using a nested PCR strategy, 51% of human oocytes and 32% of embryos exhibited mtDNA rearrangements. Multiple rearrangements were detected in 31% of oocytes and 14% of embryos. However, the important question is that of mutant load rather than frequency in cohorts, which if occurring at a very low level, may be of no biological or clinical significance. Thus, while an apparent reduction in both single and multiple mtDNA rearrangements is found in embryos compared to oocytes, it remains unclear whether this is simply due to an experimental bias related to the embryos available for analysis in clinical IVF programs, which are largely those characterized as developmentally nonviable. Collectively, current findings strongly suggest that this deletion is not a suitable indicator of reproductive senescence. Issues that need to be addressed in relating mtDNA and competence in normal women are whether suitable mutants exist and at what

load do they become problematic. Perhaps normal non-human primates oocytes may provide a model system for such studies.

THE BOTTLENECK THEORY

Rapid changes in mtDNA variants between generations have led to the ‘bottleneck’ theory, which proposes a dramatic reduction in mtDNA numbers during early oogenesis (Jansen and DeBoer, 1998). It has long been suspected that even if mtDNA mutations occur in human oocytes, a mitochondrial “bottleneck” would filter out these mutations so the resulting offspring would have no mutational load. According to this hypothesis, the expected frequency and percentage of mutated mtDNA in human blastocysts should be low or possibly absent when compared to the oocyte, and that a large percentage oocytes with high mtDNA mutational loads would simply die or not develop into embryos if fertilized. It would be interesting to evaluate the occurrence and copy number of mtDNA mutations, such as the deleted KSS mutation, in the same individual during the preimplantation, fetal and neonatal stages. In order for a species to be successful, damaged mitochondrial genomes should be eliminated to avoid a deleterious accumulation of mtDNA mutations that could be associated with a general decline in ‘cytoplasmic health’ in both oocytes and somatic cells.

This theory, however, is confounded by the presence of heteroplasmy that has been found to change rapidly in humans, sometimes in one generation. In one family with the mtDNA T8993G mutation, for example, the somatic cells of the mother had undetectable mutant mtDNA, while the mutant load in her oocytes was >75% (Blok et al, 1997). For the human, quantitation of the mutant load in the oocyte rather than in the somatic cells of the mother may be the essential issue as is the question of threshold levels of each mutation before fertility treatment may require donor oocytes. This is substantiated in the case of the mtDNA T8993G mutation since there is a strong correlation between mutant load and symptom severity of this mitochondrial disease (Blok et al, 1997) where highly skewed heteroplasmy levels in the oocytes of affected woman suggests that the mtDNA from a small number of the mother’s mitochondria (perhaps one or two) was selectively replicated to populate the offspring. However, one of the central questions that remain to be addressed in the human species is why certain developmentally lethal mtDNA mutations that accumulate in the oocyte are not eliminated during waves of apoptotic atresia that precede the establishment of the relatively small pool of gametes that exists at the onset of puberty. Perhaps mtDNA defects and mutations do not produce the types of signals that would normally trigger apoptosis in differentiated cells.

MUTATIONS THAT EFFECT MITOCHONDRIAL REPLICATION MAY BE ASSOCIATED WITH DEVELOPMENTAL COMPETENCE

The discovery of an age-dependent accumulation of point mutations in the control region of the mitochondrial genome responsible for replication raises some interesting questions concerning developmental competence and maternal age. Most strikingly, a T414G transversion was found in a high proportion (up to 50%) of mtDNA genomes in individuals above 65 years of age but was absent in younger individuals. Recently it has been found that two point mutations (A189G and T408A) within the Displacement loop (D-loop) region, the control region for mtDNA replication, occur in skeletal muscle from aged individuals (Wang et al., 2001). We have recently begun to investigate oocyte-specific mutations in the control region of the mitochondrial genome, one of which predominates in oocytes from women of advanced reproductive age (Barritt et al., 2000a, b). This mutation represents a single base pair transversion of a thymine (T) to guanine (G) at base pair 414 (T414G) in the mitochondrial genome. In women <37 years of age, the frequency of this mutation was 4% compared to 40% in the oocytes of women >37 years of age (Barritt et al., 2000a,b). Interestingly, age-dependent accumulation of this point mutations in the mitochondrial control region may be responsible for impaired transcription and regulated replication. Although there is no current evidence to suggest that this or other mtDNA mutations impair or influence the rate of mitochondrial replication after implantation, these results do provide a potentially new insight into mitochondrial mutations and aging in the human female gamete that warrants continued investigation in both fertile and infertile women, as well as with appropriate primate models.

IS THERE A RELATIONSHIP BETWEEN MITOCHONDRIA, MATERNAL AGE AND INFERTILITY

It has long been speculated that mitochondria may contribute to human reproductive senescence in older women, especially with respect to oocyte and embryo developmental competence. A high rate of generation of reactive oxygen species (ROS) and a declining capacity for oxidative phosphorylation (OXPHOS) characterize age-dependent changes in mitochondria in somatic cells where these organelles are fully developed and functional, and where these cellular components are thought to be the primary source of damaging superoxide radicals. In somatic cells, elevated levels of ROS and multiple mtDNA deletions have been associated with a variety of pathological conditions including cancer. While it has often been suggested that a similar

relationship exists in mammalian oocytes and embryos, to date, there is no direct or definitive evidence to support such a relationship. Because the oocyte is a non-dividing cell in which meiotic arrest may persist for over 45 years, it is often proposed it is vulnerable to oxidative damage and accumulation of mtDNA mutations while still resident in undeveloped and unstimulated follicles. However the oocyte is completely surrounded by and dependent upon granulosa cells for its entire life, and it is therefore reasonable to ask whether mtDNA in these somatic cells may also undergo age-related alteration. Seifer et. al. (2002) examined this question by investigating whether mtDNA deletions were more prevalent in women of advanced reproductive age by screening granulosa cells for the 4977-bp deletion. The findings suggest that the ovarian granulosa cells women over the age of 38 have a substantial decrease in the level of normal mitochondria when compared to women ≤ 34 years. However, this was a qualitative rather than quantitative analysis, and the same caveats concerning the biological significance of detection at extremely low levels discussed above for oocytes and embryos apply to these somatic cells.

It is well-known that a progressive decline in female fertility begins in the early 30s and becomes the most significant factor with respect to outcome by age 40. It is important to note that reproductive senescence may be equivalent to the peri-menopausal period at which time relatively few oocytes remain in the ovary. Numerous studies of the chromosomal normality of human oocytes show a clear increase in the frequency of aneuploidy with advanced maternal age. For example, a recent study by Kuliev et al. (2003) found that 52.1% of the 8382 oocytes from IVF patients of advanced maternal age (mean 38.5 years) were aneuploid. Whole genome amplification and single cell comparative genome hybridization to assess chromosome copy number in human embryos has revealed that more than half exhibit chromosomal abnormalities (Wells et al., 2000). It is tempting to speculate that mitochondrial defects that may influence ATP production could also influence the normality of meiotic metaphase I and II spindle assembly which in turn could lead to chromosomal malsegregation and oocyte and embryo aneuploidy. However, confirmation of this notion will require the identification of mitochondrial dysfunctions that could adversely influence cytoplasmic physiology and the dynamics of spindle assembly and disassembly.

IS THERE A RELATIONSHIP BETWEEN THE TRANSMISSION OF SPERM MITOCHONDRIAL DNA AND EMBRYO COMPETENCE?

Mammalian mtDNA has traditionally been thought to be maternally inherited with sperm mitochondria selectively destroyed by ubiquination during early embryogenesis (Uehara et al., 1976; Sutovsky et al., 2000) and diluted with oocyte mitochondria that occur in vast excess. Sutovsky et al. (2003) suggested that prohibitin may be one of the substrates that mark sperm mitochondria for elimination by proteolytic enzymes. In mice, paternal transmission of mtDNA in first-generation offspring has been observed after interspecific crosses, and the persistence of paternal mtDNAs has been detected in some *abnormal* human blastocysts (Kanada et al, 1995; St. John et al., 2000) and polyploid embryos. Despite the incompetence of these embryos, it may be relevant to ask what cytoplasmic conditions are related to the persistence of paternal mitochondria. Another possibility suggested to account for the absence of paternal mtDNA in human offspring is segregation into extra-embryonic tissues that develop into the placenta. However, no viable hypothesis has been advanced to explain how such a selective mechanism could operate. While transmission of paternal mitochondria may be more common in so-called "poor quality" oocytes and embryos, it remains to be determined whether detection could be a useful marker of competence (St. John et al., 2001; St. John et al., 2002). While the occurrence of paternal mtDNA is often dismissed as irrelevant, the use of more invasive techniques in clinical IVF such as intracytoplasmic sperm injection (ICSI), ooplasm transfer and nuclear transfer may alter the strict maternal inheritance of mtDNA. Consider the case of a case of a 28-year old man with a severe mitochondrial myopathy due to a novel 2-bp mtDNA deletion in the mND2 gene that encodes a subunit of the enzyme complex of the mitochondrial respiratory chain. The mtDNA harboring this mutation was paternal in origin and accounted for 90 percent of the patient's muscle mtDNA (Schwartz, et al., 2002). The occurrence of this patient could be an exceptional or unique situation resulting from the survival of some sperm mitochondria that ordinarily would have never been recognized had the pathogenic mutation not conferred an apparent selective proliferative advantage. While this case is assumed to be atypical, it is also apparent that underlying mechanisms responsible for elimination of sperm mtDNA in normal embryos are currently not well understood and as a consequence, further investigation of paternal mtDNA transmission and inheritance is critical if certain methods of nuclear or cytoplasmic transfer are to be used in assisted reproduction with any assurance of safety. There is increasing evidence that mtDNA anomalies in sperm may be associated with infertility. For example, mitochondrial point

mutations and deletions have been correlated with poor sperm quality (May-Panloup et al., 2003). It is unknown whether such sperm if introduced into an oocyte by ICSI are eliminated in a fashion comparable to their normal counterparts, but if not, it is an open question as to whether mitochondrial diseases could be unknowingly transmitted by this method of fertilization.

IS THERE A RELATIONSHIP BETWEEN MITOCHONDRIAL ORGANIZATION, METABOLISM AND ATP CONTENT?

Active mitochondria in several species undergo spatial redistribution in the maturing oocyte and newly fertilized embryo. Detailed studies with hamster oocytes and early embryos reveal a pattern of mitochondrial migration to the perinuclear region that persists through early cleavage stages. Although the functional significance of this relocation is unknown, it appears to be an important part of normal development in hamster embryos (Tenneille et al., 2001). A similar pattern of stage-specific mitochondrial redistribution was originally described for the mouse oocyte by Van Blerkom and Runner (1984) and later found to be mediated by arrays of microtubules originating from perinuclear microtubular organizing centers (Van Blerkom, 1991). Treatments that disrupt embryo development *in vitro* (such as the presence of inorganic phosphate or alteration of intracellular pH; Ludwig et al, 2001; Squirrell et al, 2001) also disrupt the normal pattern of mitochondrial distribution suggesting the involvement of microtubules and microfilaments. There are several reasons to believe that the clustering of mitochondria is both a normal event and an important marker for embryo competence. The relocation of the mitochondria to the perinuclear region in early hamster embryos occurs both *in vivo* and *in vitro* (Bavister et al., 2000; Ludwig et al, 2001; Tenneille et al., 2001), and has been associated with the ability of the embryos to develop *in vitro* as culture conditions which disturb the normal spatial remodeling of mitochondrial perturb or block embryo development. For example, altering intracellular pH effects the distribution of the mitochondrial clustering and disrupts perinuclear translocation (Bavister et al., 2000; Tenneille et al., 2001) and is associated with developmental arrest. Similarly, culture medium with low magnesium and high calcium concentrations that increase levels of intracellular free calcium impair the normal stage-specific redistribution of these organelles. Intracellular calcium regulates important functions such as cell division, membrane fusion, intercellular communication and metabolism, and in the hamster, disturbances in calcium homeostasis may impair mitochondrial distribution (Lane et al., 1998).

While the functional significance of stage-specific mitochondrial distribution is unknown, it is clearly associated with developmental competence in rodents, domestic species, as well as primates. Spatial

reorganizations of mitochondria have been identified in bovine and human oocytes and early embryos. When bovine oocytes are matured in vitro in the presence of glucose and amino acids, mitochondria become located primarily in the center of the oocyte, a pattern correlated with ability to develop to the blastocyst after IVF (Krisher and Bavister, 1998; Stojkovic et al., 2001). One cell human (Van Blerkom et al., 2000) and rhesus embryos (Bavister unpublished results) exhibit a pronounced perinuclear clustering of mitochondria. In human pronuclear embryos, there may be specific patterns of mitochondrial distribution indicative of embryo competence (Bavister et al., 2000; Van Blerkom et al., 2000), which suggests that noninvasive determinations of mitochondrial distribution may provide clinically relevant insight into oocyte or embryo competence.

One of the greatest challenges for research using human and nonhuman primate oocytes and embryos is to maximize the information obtained from each experiment. Epifluorescence and confocal microscopy with specific fluorescent labels can be used for imaging subcellular components but require exposure of specimens to high-energy, short wavelength light that may cause levels of cellular damage that preclude subsequent development. In contrast, multiphoton laser scanning microscopy does not appear to adversely effect development and as a result, it may be possible to visualize the distribution mitochondrial in individual oocytes and preimplantation embryos in vitro by a noninvasive method. With dual-photon microscopy used at appropriate wavelengths to excite mitochondria-specific fluorescent probes, the dynamics of mitochondrial distribution have been examined in real-time because this approach allows the correlation of mitochondrial autofluorescence in hamster and rhesus monkeys (Bavister and Squirrell, 2000) in the infrared (Squirrell et al., 1999; Bavister et al., 2000). In the rhesus monkey, mitochondrial distributions were monitored during and after fertilization in the same oocytes (Squirrell et al., 2003). Although the sample size was small in this study, this approach allows precise correlations to be made between the distribution of mitochondria and the developmental competence of the oocyte. It remains to be determined whether this method will be useful for mammalian embryos in general, and in particular, whether clinical applications exist, assuming that correlations between patterns of perinuclear clustering and developmental competence are confirmed.

The relationship between metabolism, ATP levels and oxygen consumption in oocytes and preimplantation stage embryos has been examined in several mammals including mice, rats, bovine, and human (Magnusson et al., 1986; Gott et al., 1990; Brison et al., 1994; Van Blerkom et al., 1995; Houghton et al., 1996; Thompson et al., 1996; Thompson et al., 2000; Trimarchi et al., 2000). Studies of perifollicular vascularity and intrafollicular dissolved oxygen in the human suggest a possible link between

intrafollicular conditions and oocyte/embryo competence (Van Blerkom, 1997; Van Blerkom et al., 1997; see Gregory, this volume). For example, the average net ATP-content of normal appearing metaphase II human oocytes can differ significantly between and within cohorts, and a higher potential for continued embryonic development and implantation could be associated with an ATP content above a critical threshold (Van Blerkom et al., 1995; Van Blerkom et al., 1997; Van Blerkom et al., 2000). The net ATP content present in early cleavage stage embryos is comparable to levels detected in the oocyte and as a result, the initial ATP content may be an important determinant of development potential of the embryo (Van Blerkom et al., 1995; Van Blerkom et al., 1997). Significant differences in ATP content between blastomeres of normal appearing and fragmented cleavage stage human embryos have been correlated with mitochondrial content (Van Blerkom et al., 2001), suggesting that the metabolic capacity of each blastomere is unique and may be a critical determinant of embryo competence. For the human, differential mitochondrial distribution at the pronuclear stage may be a proximate determinant of the equivalence of mitochondrial inheritance between blastomeres that be directly related to the competence of each cell (Van Blerkom et al., 2000).

In the preceding passages, I have described the potential associations between embryo competence and mitochondria with a bias towards metabolism. However, one apparent conclusion from this review is that while these organelles are a major component of the oocyte and embryo, an understanding of their precise involvement in the establishment of competence is incomplete, and they may have roles in early development beyond that of ATP production. For example, Van Blerkom et al (2002) suggested that mitochondria in human oocytes and preimplantation embryos may be heterogeneous with respect to their state of polarization and that such differences may reflect differential metabolism or participation in the regulation of intracellular free calcium. While evidence from several species demonstrates stage-specific mitochondrial redistribution occurs, the developmental function(s) of this dynamic activity has yet to be clearly established. It is also apparent that a clear correspondence between mtDNA defects and competence remains to be demonstrated, and the ability of embryos with inherited mtDNA mutations to develop to term has to be considered in the context of their known effects on mitochondrial metabolic function. Whether mammalian embryos, including the human, have the capacity to compensate for their reduced mitochondrial function by upregulating glycolysis remains to be determined. However, the effectiveness of such compensation is likely to be quite limited in view of the much lower efficiency of ATP generation from glycolysis versus oxidative phosphorylation. The relationship between mtDNA copies and the size of the mitochondrial complement needs further investigation, especially with respect

to the biological and developmental significance of reported differences between oocytes of well over an order of magnitude.

Perhaps the most important aspect of an incomplete understanding of the role of mitochondrial in the oocyte and early embryo relates to clinical activities in IVF where changes in culture media composition or invasive manipulations at the nuclear and cytoplasmic levels are undertaken in the hope of improving outcome. The expansion of donor mitochondria after cytoplasmic transfer and the persistence of paternal mtDNA are clearly unexpected findings and it is simply unknown whether these phenomena could have any adverse consequences. Likewise, as more is known about metabolic and nonmetabolic activities in which these organelles are engaged, changes in oocyte and embryo culture media and conditions may be refined to account for possible mitochondrial functions that may have different regulatory roles in early development. In this respect, one conclusion for human IVF that may be drawn from this review is that continued basic research on mitochondria is necessary and central to any effort that contemplates the manipulation of oocytes or embryos in order to improve outcome.

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CHAPTER TWELVE

FUNDAMENTALS OF THE DESIGN OF CULTURE MEDIA THAT SUPPORT HUMAN PREIMPLANTATION DEVELOPMENT

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INTRODUCTION

Human preimplantation embryos were first cultured from the zygote to the sixteen cell stage by Edwards et al. (1970). Later, human zygotes were cultured *in vitro* to the blastocyst stage (Steptoe et al., 1971). This pioneering work enabled Steptoe and Edwards (1978) to produce the first test-tube baby (Edwards, 1981). Edwards et al. (1981) summarized the media used in this early work as follows:

“Our experiences with human ova indicate that they tolerate a wide variety of culture media. The most suitable and simple medium for fertilization *in vitro* is, perhaps, Earle’s solution with the addition of pyruvate (1.1 mg/ml) and inactivated human serum (between 5% and 10%, v/v). The same medium can be used for cleavage, with higher concentrations of serum (15%, v/v), and embryos grown under these conditions will develop to term in the mother (Edwards et al, 1980). Ham’s F10 is unnecessary, although it will similarly sustain the growth of embryos capable of development to term.”

Earle’s balanced salt solution was designed for studies on mouse fibroblasts *in vitro* (Earle, 1943), and Ham’s F10 was designed for the culture of Chinese hamster ovarian cells and mouse L cells (Ham, 1963). It was soon recognized, however, that while these media supported the initial cleavage divisions of human preimplantation embryos over two or three days of culture, they poorly supported development for a further two days to the blastocyst stage (see review: Bolton and Braude, 1987). Since then, two new strategies have been introduced for the culture of human preimplantation

embryos: firstly, the introduction of chemically defined media in order to eliminate the need for serum supplements, and secondly, the introduction methods whose objectives were to increase the yield of blastocysts, either by the use of co-culture or sequential culture procedures. Methods involving the sequential use of two media now dominate the field. The objectives of this chapter are: (1) to focus on how the current status of the field evolved, and (2) to analyze critically the evidence on which the composition of current media is based. No attempt will be made to cover the extensive, frequently confusing, literature on the development of media for the culture of human and non-human embryos. Much of the literature has been described in detail in several reviews (Biggers, 1987, 1998, 2001; Gardner, 1994; Bavister, 1995; Gardner and Lane, 1999; Gardner et al, 2000; Loutradis et al, 2000; Martin, 2000; Quinn, 2000; Lane, 2001; Smith, 2002).

SOME DEFINITIONS

A **biological medium** is one that contains a body fluid or an extract of some tissue, organ or embryo, such as blood serum and embryo extract. The chemical composition of such a medium is unknown. The composition of a *chemically defined medium*, as the name implies, is known. It is composed of a set of chemical constituents, not necessarily pure, dissolved in water. This idea of using chemically defined media for the culture of cells was first proposed by Lewis and Lewis (1911a,b, 1912), only four years after it was initially shown that cells, specifically nerve cells, could be grown *in vitro* (Harrison, 1907). The reasons for designing chemically defined media were pragmatic. They were stated by Lewis and Lewis as follows:

- (i) the media can easily be reproduced at different times
different laboratories
- (ii) the media can be varied in a controlled manner
- (iii) the media are free of unknown enzyme activities,
and hormones and growth factors, which may interfere
with the responses being studied.
(italics are author's amendment).

Note that the definition of a chemically defined medium refers to its composition at the beginning of an experiment involving culture. The fact that the composition of the medium may be changed during the culture period due to interactions with the explanted cells being cultured does not invalidate the definition.

Recently the term *protein-free medium* has been introduced. These are chemically defined media that are devoid of proteins isolated from natural

sources. The development of such media has been driven because of the concern that these proteins may transmit disease, such as those caused by viruses and prions.

EARLY HISTORY

The design of chemically defined media became a serious area of research in the 1940s with the publication of the compositions of media by White (1946) and Fischer (1947) for plant and animal cells. In the next decade several other chemically defined media were introduced (for review see: Biggers et al, 1957; Waymouth, 1965, 1972). A medium that has become significant for the culture of human preimplantation embryos is Eagle's minimum essential medium (MEM) which was designed for the culture of several human cell lines (Earle, 1959).

In the early work on the culture of human preimplantation embryos, serum, usually serum from the patient providing the oocytes, was added to either Ham's F10 medium or Earle's physiological saline, thus converting them into biological media. The possibility that media could be developed for the culture of human preimplantation embryos that are serum-free, and thus chemically defined, was demonstrated by Menezo et al. (1984). In the following year another chemically defined medium was described (Quinn et al, 1985), named human tubal fluid medium (HTF). This medium should not be confused with a chemically defined medium called synthetic tubal fluid (STF), which was introduced for physiological studies on human sperm motility and capacitation (Mortimer, 1986). More recently, media of unknown composition which are claimed to be totally free of proteins have been reported (Ali et al, 2000).

In most of the work done on the treatment of human infertility using *in vitro* fertilization prior to 1987, ova were cultured for 2 days to about the 8-cell stage and then returned to the patient. The adoption of this procedure was due largely to the fact that all the available media only supported the development of blastocysts in a very low yield, some of them damaged. The first attempt to improve the production of blastocysts by extending culture to five days involved the use of co-culture (Menezo, 1987; Menezo et al, 1990). This technique, although effective, creates an environment of unknown composition. Co-culture has now been largely abandoned in favor of the use of pairs of media used sequentially for the *in vitro* production of human blastocysts (Menezo et al, 1998; d'Estaing et al, 2001). The use of paired media in sequence has been successfully championed by Gardner and Lane (1997) and Gardner (1998). At present, there are at least eight commercially available pairs of media for the extended culture of human preimplantation embryos.

The culture of the preimplantation embryos of laboratory and domestic species has been an active area of research for decades. Work on the mouse, which has been recommended as a model for the human (Quinn and Horstman, 1998), has had a major impact on the design of media for the culture of human preimplantation embryos. In 1949 Hammond (Hammond, 1949) first successfully cultured mouse preimplantation embryos through part of their development in a biological medium containing egg white and egg yolk in a phosphate-buffered medium. A few years later, in 1956, a chemically defined medium was designed (Whitten, 1956) which supported the development of the eight-cell mouse embryo into a blastocyst. This medium was a chemically defined physiological saline (Krebs-Ringer bicarbonate), designed by Krebs and Henseleit (Krebs and Henseleit, 1932) for the study of tissue metabolism, supplemented with bovine serum albumin (BSA) and glucose. Thus, this simple medium contained only eight constituents dissolved in water. In 1958 McLaren and Biggers used this medium to produce blastocysts which later developed into newborn young after they were transferred to the uterus of a surrogate mother (McLaren and Biggers, 1958). As a result of replacing calcium chloride with calcium lactate in his initial medium, Whitten (1957) discovered serendipitously that mouse embryos would develop from the two cell stage to the blastocyst. Further studies eventually showed that the mouse oocyte and one cell stage were dependent on the presence of pyruvate in the medium (Biggers et al, 1967). This dependence on pyruvate seems to be universal among mammalian species and is included in all media for the culture of human preimplantation embryos.

In the next few years several variants of Whitten's medium were proposed for the culture of mouse preimplantation embryos (for review see: Biggers, 1987). One of these media was described by Brinster (Brinster, 1963). The hope that these media would support the development of the preimplantation embryos of other species was soon questioned when Purshottam and Pincus (1961) reported that rabbit embryos would not develop in Whitten's medium but required a more complex medium. Kane and Foote (Kane and Foote, 1971; Kane et al, 1997) subsequently designed a medium by supplementing a slightly modified Brinster's medium with several classes of compounds found in Ham's F10 (amino acids, vitamins, trace elements, nucleic acid precursors) to produce a complex medium that would support rabbit development. In the historical context of clinical IVF, the early work on the culture of the preimplantation human embryo made little use of these media developed for the mouse and rabbit. Presumably the embryologists and clinicians involved did not want to undertake the tedious work of making up these media when media such as Earle's balanced salt solution and Ham's F10 were commercially available. These two media are still frequently used.

OVERALL DESIGN OF CHEMICALLY DEFINED MEDIA

There are two major problems in the design of chemically defined media. The first is the selection of compounds to include. The second is the determination of the concentrations of each compound. Figure 12.1 is a Venn diagram showing the classes of compounds that are found in chemically defined media for the culture of mammalian embryos. The compounds consist mainly of components common to all body fluids. A few of them may be specific to the oviduct fluid and a few are artificial chemicals not found naturally. The number of compounds may be small to give the arbitrarily defined “simple” media which only contain ~12 constituents, or “complex” media containing many more than 12 compounds.

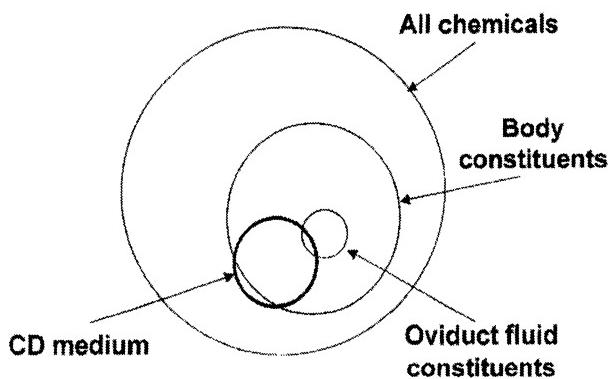


FIGURE 12. 1. The classes of compounds included in media for the culture of preimplantation embryos. (from Biggers, 2002).

The compositions of most media are still largely based on one of three physiological salines: Earle's balanced salt solution (Earle, 1943), Krebs-Ringer bicarbonate (Krebs and Henseleit, 1932), and Tyrode's solution (Tyrode, 1910). Tyrode's solution is the basis of Whittingham's medium M16 (Whittingham, 1971), that has been used for the culture of mouse preimplantation embryos for many years. These physiological salines are supplemented by varying numbers of compounds from the two different classes of compounds found in the body fluids. Examples of added substances in the third class, which do not occur naturally, are EDTA, antibiotics, and the

pH indicator phenol red. Growth factors are known to be involved in early mammalian development, and some are of oviductal origin (Kane et al, 1997; Buhi et al, 2000; Hardy and Spanos, 2002), but so far these have not been routinely incorporated into media for the culture of preimplantation embryos.

Two approaches have been used to determine the concentrations of components used in a medium: (1) “let the embryo choose” principle, and (2) the “back to nature” principle. Using the “let the embryo choose” approach, a bioassay is done in which the response of cells to several concentrations of a component is measured. The results are used to estimate a concentration-response line. The concentration selected for use in a medium is usually that which gives a maximum response. The “back to nature” approach uses the concentration of a substance which is present in the natural environment of the embryo.

LET THE EMBRYOS CHOOSE PRINCIPLE

A traditional approach has been to vary the concentrations of each compound separately, keeping the concentrations of the other components constant. An example of this strategy where the percentage of blastocysts that develop from two-cell mouse embryos in media with different osmolarities is shown in figure 12.2 (Biggers and Brinster, 1965). The fitted regression line rises to a maximum as the osmolarity is increased, after which it falls. The estimated maximum is 277 mosmols. On the basis of “let the embryo choose” all media for the culture of mouse preimplantation embryos should have an osmolarity ~ 270 mosmols.

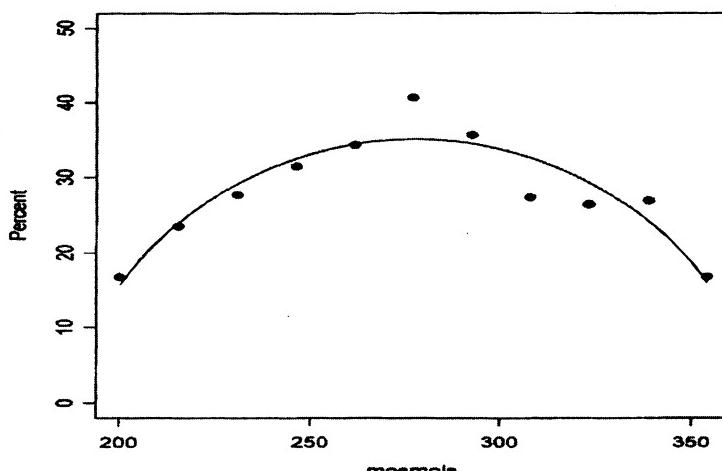


FIGURE 12.2. Effect of osmolarity on the development of two-cell mouse ova into blastocysts. (calculated from the data of Biggers and Brinster, 1965).

The design of a culture medium, however, involves the simultaneous choice of all the concentrations in a mixture because the effects of each of the components of the medium may depend on the concentrations of the other components. This problem can be illustrated geometrically by representing the joint action of two components as a concentration-response surface in three-dimensional space (Figure 12.3). The graph shows clearly that the response to Compound 1 is very dependent on the concentration of Compound 2. Thus, the study of each compound in isolation would provide very incomplete information on which to select the concentration(s) that stimulates a maximal response. In the case of a medium containing more than two constituents, say n components, the concentration-response surface can be represented in $(n+1)$ -dimensional space. This model, proposed by Biggers et al. (1957), in a discussion of the design of media for cell and organ culture, was based on a similar model introduced by Box (1957) to approximate the yields in a complex chemical engineering process.

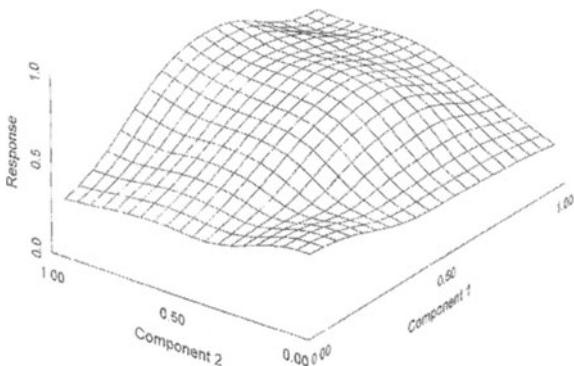


FIGURE 12.3 Two-dimensional concentration response surface. (from Biggers, 2002).

To explore a concentration-response surface, experiments that vary the concentrations of only a single factor must be replaced by other experimental strategies, such as factorial, fractional factorial, rotatable and shell designs, in which the number of concentrations of each component is >2 . For example, a 3^3 factorial design was used to investigate the joint actions of NaCl, glutamine and glucose (Lawitts and Biggers, 1992). These experimental designs become intractable when the numbers of components in a medium is >3 . To explore the concentration-response surface associated with a medium containing 12 constituents, each at three concentrations, would require the comparison of $3^{12} = 531,441$ media! An alternative strategy is to use a method which locates the

maximum point on the concentration-response surface. This point is sometimes called the ***optimum response***. It should be noted, however, that the maximum response is not necessarily the ***natural response***. Several procedures have been described for finding the maximum of a regression surface (Everitt, 1987)). One method is sequential simplex optimization (Spendley et al, 1962; Walters et al., 1991)).

Sequential simplex optimization was used to design a medium for the culture of mouse preimplantation embryos through a block to development, called the two-cell block, which frequently interfered with studies on early development (Lawitts and Biggers, 1991, 1992). This medium was called medium SOM. The medium was subsequently modified as the result of measurements of the intracellular concentrations of some inorganic ions in the blastomeres of two-cell mouse embryos exposed to SOM (Biggers et al, 1993) to give the medium called KSOM (Lawitts and Biggers, 1993). Although KSOM medium was based on the proportion of embryos that passed through the two-cell block, it fortuitously proved very effective for the production of blastocysts from the zygote (Erbach et al, 1994).

BACK TO NATURE PRINCIPLE

The “back to nature” approach proposes that the concentration of a substance incorporated into a medium should approximate the concentration to which the embryo is naturally exposed. The principle was first invoked to design a medium for the culture of the sheep preimplantation embryo (Tervit, 1972). Quinn et al. (1985) subsequently used this principle to design a medium for the culture of the human preimplantation embryo called medium human tubal fluid (HTF).

The “back to nature” approach depends on prior knowledge of the compositions of the female genital tract fluids in the oviduct and the uterus. Unfortunately, knowledge of the composition of the oviductal and uterine fluids *in situ* is far from complete (for reviews see: Leese, 1987; Leese et al, 2001) because of the limited volumes available for chemical analysis. The difficulties associated with some of the early methods for collecting oviductal fluid have been listed by Restall (1966) as follows: (1) ligation of the oviduct inhibits the secretion of fluid by raising the intraluminal pressure, (2) flushing of the tract precludes the determination of concentrations, (3) the mechanical extrusion of the fluid may contaminate the fluid with cellular debris, and (4) the collection of fluid post-mortem may be effected by ionic shifts after death. Similar difficulties arise in the collection of uterine fluid for analysis. Restall (1966) attempted to avoid the difficulties of collecting oviductal fluid by using a cannulation technique, collecting the fluid over several hours. This method, unfortunately, will obliterate any regional differences of the

secretions along the oviduct and is subject to degradation of the constituents. Holmdahl and Mastroianni (1965) attached a small refrigeration unit to the collection catheter to minimize such degradation.

In recent years, the development of ultramicrochemical methods has allowed the sampling of small volumes of oviductal fluid for chemical analysis using micropuncture or microsampling techniques in the mouse (Roblero et al, 1976; Borland et al, 1977; Gardner and Leese, 1990) and human (Borland et al, 1980; Gardener et al, 1996). Microsampling of genital tract secretions at known times overcomes many of the objections to the older techniques, and has provided information on the concentrations of Na^+ , K^+ , Cl^- , Ca^{2+} and Mg^{2+} , glucose, pyruvate and lactate in human oviductal fluid (Table 12.1). Microsamples of human uterine fluid have been aspirated from the uterine cavity (Casslen and Nilson, 1984; Gardener et al, 1996), enabling the measurement of the concentrations of Na^+ , K^+ , Ca^{2+} , Cl^- , pyruvate, lactate and glucose.

Ideally, the concentrations of constituents in the local microenvironments of developing preimplantation embryos, as they journey from the ampullary region of the oviduct to the uterus, need to be known for the design of suitable chemically defined media. This requirement has been met by Borland et al. (1977) in their studies on the mouse. Such samples cannot be obtained from women, largely for ethical reasons. As a substitute an heuristic approach has been used in which samples of fluid from patients with normal menstrual cycles are obtained at different stages of the cycle. The assumption is then made that the concentrations found in the luteal phase approximate those that the preimplantation embryo experiences. Thus, Borland et al. (1980) obtained microsamples of ampullary fluid from seven patients undergoing hysterectomy and bilateral salpingo-oophorectomy for menometrorrhagia. High concentrations of K^+ and Cl^- and low concentrations of Ca^{2+} compared to the concentrations found in serum were observed, but no differences were detected in the concentrations of Na^+ and Mg^{2+} . Microsamples of ampullary and uterine fluids obtained from patients undergoing laparotomy during treatment for infertility (Gardner et al, 1996) showed that the concentrations of pyruvate and lactate were higher in the fluids recovered from the ampullary region of the oviduct than fluids obtained from the uterus, while the concentration of glucose was lower. Casslén and Nilsson (1984) obtained microsamples of uterine fluid from 129 patients in the proliferative, midcycle and luteal phases who "desired contraceptive counseling". The concentration of glucose was not different from that in serum and did not change with the phases of the menstrual cycle. The concentrations of K^+ was higher and Ca^{2+} lower than in serum. Both varied cyclically being lower during the midcycle phase than in the proliferative and luteal phases. The Na^+ concentration was

lower than in serum and did not change with the phases of the menstrual cycle.

In designing medium HTF, Quinn et al. (1985) based the concentrations of some of the constituents of this medium on the concentrations in the oviduct reported by Lippes et al. (1972) and Lopata et al. (1976). The analyses of Lippes et al. (1972) were on samples obtained over an extended period of time from cannulated oviducts, while the analyses of Lopata et al. (1976) were based on microsamples from two human patients. The concentrations of only three ions (Na^+ , K^+ , Ca^{2+}) were measured in both of these studies. There were considerable differences in the concentrations of all three ions in the two studies (Table 12. 1). The composition of HTF is also shown in Table 12.1 together with other published analyses of human oviductal fluid. It is clear that, with few exceptions, there is only a slim agreement between the composition of HTF and the composition of human oviductal secretions. Furthermore, there are only eight constituents in HTF which are only a very small subset of the compounds in oviductal fluid. Although HTF supports early development of human preimplantation embryos *in vitro*, it is potentially misleading to name it “human tubal fluid”.

TABLE 12.1. COMPARISON OF THE COMPOSITION OF MEDIUM HTF WITH PUBLISHED ANALYSES OF THE COMPOSITION OF HUMAN OVIDUCTAL FLUID
[modified from Biggers, 2002].

Compound (mmol/l)	Lippes et al. (1976) ^a	Lopata et al. (1976) ^b	Medium HTF ^c (1985)	David et al. (1973) ^d	Borland et al. (1980) ^e	Gardner et al. (1996) ^f
Na^+	139-140	149.2	148.3	142-148	130	-
K^+	7.7-9.9	4.5	5.06	6.7	21.2	-
Cl^-	117-120	-	108.3	112-127	132	-
Ca^{2+}	3.8-4.8	1.38	2.04	-	1.13	-
Mg^{2+}	-	0.19	0.2	-	1.42	-
Glucose	2.39-3.04	-	2.78	-	-	2.32
Pyruvate	-	0.18	0.33	-	-	0.16
Lactate	-	2.52	21.4	-	-	6.19

^a Collected over 24 h by cannulation (16 specimens) (Lippes et al., 1972).

^b Collected at laparoscopy (two specimens) (Lopata et al., 1976).

^c Concentrations of ions calculated from the concentrations of constituents in Table 7 (Quinn et al., 1985).

^d Collected at laparotomy (33 specimens) (David et al., 1973).

^e Microsamples collected by microsampling (seven specimens) (Borland et al., 1980).

^f Microsamples collected during the luteal phase at laparoscopy (nine specimens) (Gardner et al., 1996).

There is evidence from studies on mice that the concentration of substances in oviductal fluid does not necessarily support the development of preimplantation embryos in a chemically defined medium. This fact was first observed in studies on K⁺. Although the mouse oviductal fluid contains a high concentration of K⁺ (~25 mmol/l) (Roblero et al, 1976; Borland et al, 1977), it was reported that mouse zygotes do not develop in concentrations ranging from 12-48 mmol/l (Whittingham, 1975). In contrast, Wales (1970) found that two-cell mouse embryos would develop in K⁺ concentrations ranging from 1-48 mmol/l, Roblero and Riffo (1986) reported that a concentration of 25 mmol/l K⁺ would support preimplantation development in the mouse, Quinn et al. (1985) found maximum development when the concentration of K⁺ ranged from 2.3-5.1 mmol/l, and Wiley et al. (1986) found that mouse embryos developed best when the K⁺ concentration was <6 mmol/l. The concentration of K⁺ in KSOM is 2.85 mmol/l (Lawitts and Biggers, 1993). Why the results are so variable is not at all clear. Could it be due to differences in the concentrations of other components in the different media being used?

Another example of the lack of correspondence between the conditions that support a maximal response *in vitro* and the natural environment concerns osmolarity. The concentration-response line in 12. 2 shows that the maximum percentage of two-cell embryos that develop into blastocysts occurs when the osmolarity of the medium is 277 mosmols. This value is significantly lower than the osmolarity the embryos experience naturally in the oviduct where the osmolarity has been estimated to fall in the range 290-300 mOs/Kg (Collins and Baltz, 1999). The reason for this discrepancy is discussed later.

A further example where the conditions that support a maximal response *in vitro* differ from the natural environment is the pH. There are several studies involving the rhesus monkey, rabbit, rat and mouse which report that the pH of the oviductal fluid ranges from 7.7-8.2 (for review see: Phillips et al, 2000). In contrast, the pH of media for the culture of mammalian preimplantation embryos, including human embryos, is usually about 7.2. The use of pH, however, can conceal the true picture in physiological analyses since it is a non-linear transformation of the relevant parameter, namely the hydrogen ion concentration (Stewart, 1978; Tyler-Jones and Taylor, 1999). When the above results are expressed as [H⁺], the concentration in oviductal fluid ranges from 6.31-19.95 nmoles/l and the optimum [H⁺] for culture is 39.81 nmoles/l, about 2-7 times higher.

The fluid that is in intimate contact with the cells of the embryo is the perivitelline fluid that lies between the vitelline membrane and the zona pellucida. No chemical analyses have been made of this fluid. A study on the permeability of the zona pellucida in the rabbit, rat and hamster showed that

molecules with molecular weights as high as 1200 readily pass through, while heparin with a molecular weight of about 16,000 does not (Austin and Lovelock, 1958). However, the fact that the Mengo encephalitis virus (diameter 27-28 m μ) can pass through the zona pellucida of mice suggests that the barrier to the passage of material is less than suggested by the results with heparin (Gwatkin, 1963). It seems likely that the concentrations of small molecules in the oviductal and perivitelline fluids are similar.

A caveat: It is important to recognize that both the "let the embryos choose" and the "back to nature" approaches to media development have limitations. The "let the embryos choose" principle determines the concentrations of a finite set of constituents that leads to a maximum response. The combination of concentrations may well be changed if the medium is supplemented with further constituents. Further, there is no guarantee that the maximum response is the natural response. The "back to nature" principle is limited by the difficulty of determining the concentrations of substances in the environment in which the embryos develop naturally. Caution should be employed in using estimates of compounds in the natural environment since there are several examples where apparent optimum concentrations *in vitro* differ from the concentrations found naturally.

STUDIES ON ANIMALS THAT HAVE IMPACTED MEDIA DEVELOPMENT

Studies done on animals in these areas have had major impacts on the development of media for the culture human preimplantation embryos. These are the investigation of the energy sources required to support development, the action of glucose and phosphate on preimplantation development, and the requirement for amino acids.

ENERGY REQUIREMENTS

The findings of Whitten (1956;1957) that glucose would not support the development of the preimplantation mouse embryo before the eight-cell stage, and that lactate would not support development before the two-cell stage, led Biggers et al. (1967) to investigate the requirements of the maturing mouse oocyte mouse and one-cell stage embryo. The combined results showed that sometime during oogenesis, the energy pathways become

restricted such that only pyruvate, and three other closely associated intermediates in the energy pathway, would support oocyte maturation and division of the one-cell embryo. As development proceeds, the energy pathways seem to open up so that by the eight cell stage, the Embden-Meyerhoff pathway is fully functional again. It was this work that led to the routine incorporation of pyruvate into media for the culture of the initial stages of development of all mammalian species. The subsequent demonstration that ultrafluorometric analytical methods were sufficiently sensitive to study single preimplantation embryos (Leese et al, 1984) showed that pyruvate was the preferred source of energy by the mouse mature oocyte and zygote, and that by the eight cell stage, glucose became the preferred source (Leese and Barton, 1984). Later, it was demonstrated in the human preimplantation embryo that the uptake of glucose relative to pyruvate increased after the eight-cell stage (Figure 12.4) (Leese et al, 1993). The uptake of pyruvate by the human preimplantation embryo, however, remained high throughout development to the blastocyst stage and does not sharply decline as in the mouse. These findings paved the way to the design of pairs of media that are used sequentially to culture human preimplantation embryos (Barnes et al, 1995).

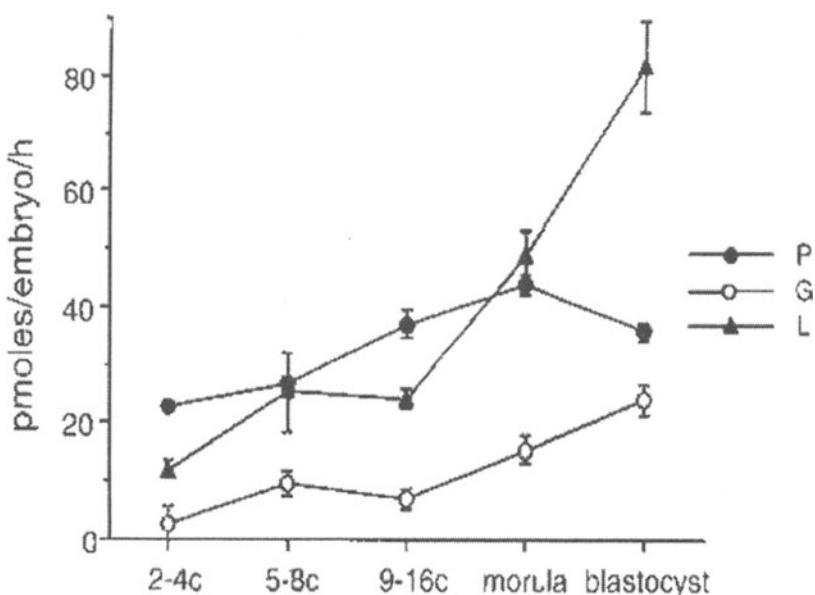


FIGURE 12. 4. Uptake of pyruvate and glucose, and the formation of lactate by single human preimplantation embryos *in vitro*. (from Leese et al., 1993, reprinted with permission).

DOES GLUCOSE INHIBIT EARLY PREIMPLANTATION DEVELOPMENT?

The observation of Schini and Bavister (1988) that glucose and phosphate caused arrest of preimplantation development of the hamster embryo at the two-cell stage had a major impact on the design of media that support human preimplantation development. The observation was soon confirmed for the mouse (Chatot, 1989), and it was a major influence on the design of medium CZB for the culture of the mouse zygote to the blastocyst. Medium CZB does not contain glucose and inorganic phosphate (P_i). The inhibitory action of glucose was subsequently described in several other species including the human (Conaghan et al, 1993; Hardy, 1994). Soon after, a modification of Quinn's medium HTF was formulated in which glucose and phosphate were omitted and EDTA and glutamine added (Table 12.2) (Quinn et al, 1995). This medium closely paralleled the composition of medium CZB. More recently another medium for the culture for human preimplantation embryos has been described called P1 (Table 12.2) (Gardner et al, 2000). This medium is, in fact, a modified form of Quinn's glucose- free and phosphate-free modified HTF medium in which citrate and taurine are substituted for glutamine and EDTA.

The need to omit glucose from culture media for the initial stages of preimplantation development seems paradoxical since the oviduct contains the compound in significant amounts (Gardner and Leese, 1990; Gardner et al, 1996). The attention paid to glucose inhibition, however, has obscured the several reports in which glucose was not found to inhibit early development. Strong evidence now exists which suggests that the background composition of the media used determines whether or not glucose inhibits early mouse development (for review see: Biggers and McGinnis, 2001). Medium KSOM supports the normal development of the mouse zygote to the blastocyst stage when the glucose concentration is raised to the level found in blood (5.56 mmol/l) (Figure 12.5). Phosphate only slightly inhibits development in KSOM, and its effects are independent of the concentration of glucose.

Unfortunately, the widespread belief that glucose and phosphate absolutely inhibit early human development is now dogma. The need to exclude glucose and phosphate or use glucose in a low concentration is touted in the advertisements of several commercially advertised media. The dogma precludes recognition of the possibility that media could be designed to support early human development in which glucose and phosphate are present in concentrations approximating their normal range. The chemically defined medium B3, specifically designed for the culture of the human preimplantation embryos, supports cleavage despite the presence of glucose (6.7 mmol/l), $Na_2HPO_4 \cdot 12H_2O$ (0.85 mmol/l) and KH_2PO_4 (0.44 mmol/l).

There is also more recent evidence that glucose does not inhibit development of the early human embryo in some media (Barak et al, 1998).

TABLE 12.2. COMPOSITIONS OF MEDIA HTF (QUINN et al., 1985), MODIFIED HTF (QUINN et al., 1995) AND P1 (GARDNER et al., 2000).

Compound (mmol/l)	HTF	Modified HTF	P1
NaCl	101.6	101.6	101.6
KCl	4.69	4.69	4.69
KH ₂ PO ₄	0.37	-	-
CaCl ₂	2.04	2.04	2.04
MgSO ₄	0.20	0.20	0.20
NaHCO ₃	25.0	25.0	25.0
Glucose	2.78	-	-
Na pyruvate	0.33	0.33	0.33
Na lactate	21.4	21.4	21.4
Citrate	-	-	0.5
Glutamine	-	1.0	-
Taurine	-	-	0.05
EDTA	-	0.1	-
Penicillin (U/ml)	100	100	-
Streptomycin SO ₄ (50mg/ml)	100	100	-
Phenol red (%)	0.001	0.001	5mg/ml

Coates et al. (1999) compared the development of preimplantation human embryos in Earle's balanced salt solution with and without 5.5 mmol/l glucose, the concentration normally found in blood. The pregnancy rates using the two media were not significantly different. They did find, however, statistically lower cleavage rates and grades of embryo in the medium containing glucose. They concluded: "... a reduction of the glucose concentration of the medium used for embryo culture from the pronucleate stage to embryo transfer on day 2 or 3 is prudent". The statistical differences were marginal and were based on large numbers of embryos. Thus, the results may have little clinical significance (Biggers and McGinnis, 2001). It is now generally accepted that glucose does not absolutely inhibit the initial stages of human preimplantation development and that glucose should be included in media at the concentrations found in the human oviduct (Gardner and Lane, 1999; Coates et al, 1999; Bavister, 1999).

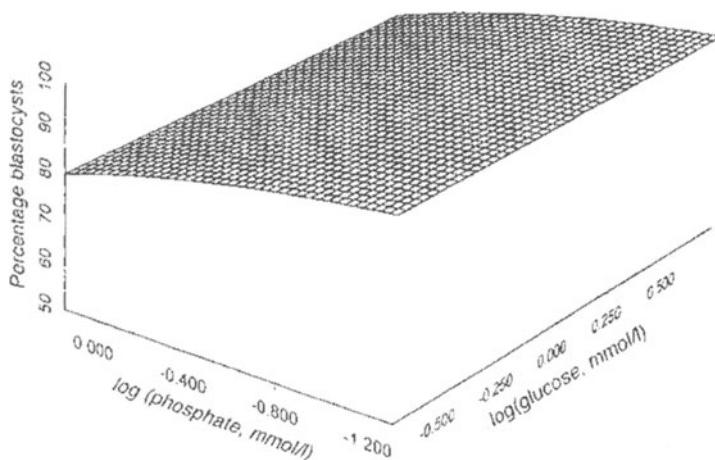


FIGURE 12. 5. A concentration response surface showing the joint action of glucose and phosphate on the development of mouse zygotes into blastocysts cultured in medium KSOM^{AA} (from Biggers and McGinnis, 2001, reprinted with permission).

REQUIREMENT FOR AMINO ACIDS

In the following discussion, “amino acids” is a generic term denoting collectively all 20 common naturally-occurring amino acids. All of these amino acids were present in some of the media used in the initial work on the culture of human embryos, namely Ham’s F10 (Edwards, 1981) and media B2/B3 (Menezo et al, 1984). The possibility that amino acids are not necessary, as is the case in the mouse (Cholewa and Whitten, 1970), arose when Quinn et al. (1985) showed that human zygotes develop in medium HTF which does not contain these compounds. Nevertheless amino acids have beneficial effects which have been recognized for many years in several species, beginning with the work of Brinster (1995a) on the mouse (for review see: Biggers, 1998; Gardner, 1994; Gardner et al, 2000; Devreker et al, 2001). The rigorous proof that amino acids influence the development of preimplantation embryos *in vitro* requires that they be tested in a base medium that does not contain other proteins, such as BSA which may also be a source of amino acids (Cholewa and Whitten, 1970; Brinster, 1965a). The effect of varying the concentrations of amino acids on the development of mouse preimplantation embryos in a base medium in which BSA was replaced with polyvinyl alcohol, was studied by Biggers et al. (1997;2000) (Figure 12. 6). Varying the concentrations of the set of all 20 common amino acids had little effect on the rate of blastocyst formation, but significantly

increased the rate of hatching, and, more importantly, significantly increased the total numbers of cells in the blastocysts, particularly in the inner cell mass. Further, the extracellular matrix in the blastocysts that developed was more normally organized when amino acids were present. These results confirmed much of the work of others on the combined effects of amino acids on the development of mouse preimplantation embryos *in vitro* where BSA was retained in the media. On reflection, it is not surprising that amino acids exert beneficial effects on the development of the preimplantation embryo since they are known to have multiple functions including: precursors for biosynthesis, energy sources, regulators of energy metabolism, osmolytes, buffers of intracellular pH, and chelators of heavy metals (for review see: Gardner and Lane, 2000).

The determination of the concentrations of each of these amino acids to include in a medium which optimizes the development of preimplantation embryos is a formidable task. Their joint effects can be theoretically represented by a concentration-response surface in 21-dimensional space. To explore this surface with a factorial experiment using three concentrations of each amino acid would require the comparison of $3^{20} = 3,486,784,401$ media! Seeking the combination of concentrations that give a maximum response using sequential simplex optimization would be logically feasible, although the procedure becomes less efficient as the number of factors to optimize increases.

Two ways have been used to determine the concentrations of amino acids to include in media. Firstly, the effects of each amino acid can be examined by supplementing a base medium with each compound individually. Secondly, the base medium can be supplemented by either the complete set or subsets of all 20 amino acids. The concentrations of amino acids used by Brinster (1965a) were based on the amino acid composition of bovine plasma albumin. Most recent work on the amino acid requirements of preimplantation embryos has utilized the so-called essential and non-essential amino acids, mixtures that are available commercially. These terms were first coined in nutritional studies of whole animals (Rose, 1938), essential amino acids being required in the diet while the non-essential amino acids are not. The same terms were adopted by Eagle (1959) to describe his findings on the nutritional requirements for amino acids of several human cell lines cultured *in vitro*. His work identified two subsets of the common natural amino acids (Table 12.3) 13 “essential” amino acids that had to be included in the culture medium and seven “non-essential” amino acids that were not required. The essential subset of amino acids was included in the widely used “minimum essential medium” (MEM), while the non-essential subset was not included in MEM. The generic use of the terms “essential” and “non-essential”, including specified concentrations of the amino acids to use in all systems, as proposed

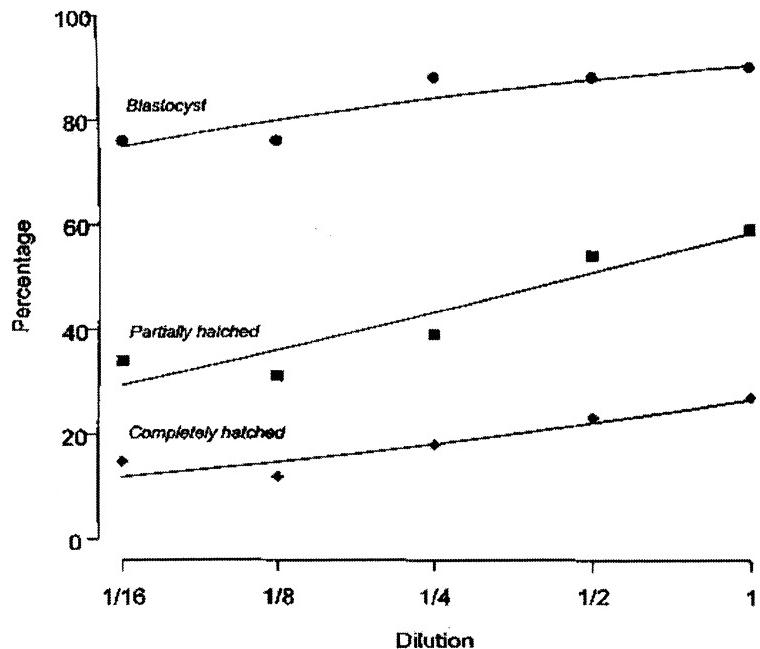


FIGURE 12.6. The effect of different dilutions of Eagle's amino acids in KSOM on the percentage of zygotes that develop at least into zona-enclosed blastocysts, and partially hatched or completely hatched blastocysts. (from Biggers et al., 2000).

by Eagle, was soon challenged, largely on the grounds that they ignore the fact that concentration-response surfaces vary according to the cells being cultured. In an extensive discussion of the term, Waymouth (1965) wrote:

“The terms “essential” and “non-essential” amino acid, rather commonly used, are not, therefore, strict designations and should be accepted with skepticism unless carefully in a particular context ...”

Since mouse and human zygotes develop into blastocysts in media that do not contain amino acids (Whitten and Biggers, 1968; Cholewa and Whitten, 1970; Biggers, 1987; Conaghan et al, 1993), none of the 20 amino acids can be considered absolutely essential. Recently, Houghton et al. (2002) have also pointed out that the classification is over-simplistic and may mask important subtle effects. The terms essential and non-essential, therefore, have little significance in the field of preimplantation embryo culture and ideally their use should be dropped.

TABLE 12.3. ESSENTIAL AND NON-ESSENTIAL AMINO ACIDS DETERMINED FOR SOME HUMAN CELL LINES (EAGLE, 1959).

Essential amino acids	Non-essential amino acids
L-arginine	L-alanine
L-cystine	L-asparagine
L-glutamine	L-aspartic acid
L-histidine	L-glutamic acid
L-isoleucine	Glycine
L-leucine	L-proline
L-lysine	L-serine
L-methionine	-
L-phenylalanine	-
L-threonine	-
L-trptophan	-
L-tyrosine	-
L-valine	-

The reason the terms essential and non-essential have become ingrained in the literature is because mixtures of Eagle's essential and non-essential amino acids have been commercially available for some time. These prepackaged mixtures have been used extensively in the preparation of media for the culture of preimplantation embryos since their use avoids the extensive labor involved in adding amino acids to a base medium separately. A severe limitation in their use in experimental work, however, is the restriction that the relative concentrations of amino acids within each of the two subsets of amino acids cannot be changed.

The most extensive studies on the role of amino acids on the development of mouse preimplantation embryos *in vitro* has been done by Gardner and Lane (reviews: Gardner, 1994; Gardner and Lane, 1999; Gardner et al, 2000). An understanding of their work is important since it has been the basis in part for the introduction of the sequential two-step method for the culture of human preimplantation embryos (see below). In initial studies, Gardner and Lane (1993) exposed mouse zygotes to medium mMTF (modified Mouse Tubal Medium) supplemented with all 20 amino acids, non-essential amino acids with or without glutamine, and essential amino acids with or without glutamine. After 96 hours of culture, the best response of the embryos, assessed morphologically and by cell number, was obtained using the non-essential amino acids. In contrast, essential amino acids were inhibitory. Also, when culture was extended for more than 96 hours, development was inhibited even in the presence of non-essential amino acids. This delayed

inhibition was due to ammonia accumulating in the medium produced by the deamination of amino acids such as glutamine. In subsequent work it was shown that the best development is obtained when zygotes are cultured in medium mMTF supplemented with non-essential amino acids and glutamine to the eight cell stage, followed by culture to the blastocyst stage in medium mMTF supplemented by all 20 amino acids (Lane and Gardner, 1997). Recently Lane et al. (2001) have shown that development was enhanced if the essential amino acid concentration was reduced to half strength. It is interesting to note that another medium, amino acid supplemented KSOM, also designed for the culture of mouse preimplantation embryos, has always contained essential and non-essential amino acids at half strength (Ho et al, 1995; Biggers et al, 2000). In recent years some attempts have been made to elucidate the role of amino acids on the development of human preimplantation embryos *in vitro*. Varying the concentration of glutamine increased the yield of blastocysts (Devreker et al, 1998). Other work has shown that taurine and glycine are organic osmolytes (Devreker et al, 1999; Hammer et al, 2000). These results, however, do not help in the determination of the optimum concentrations to use in culture media.

Studies using the non-essential and essential amino acid mixtures have resulted in two-step techniques for the culture of human embryos similar to that used for the culture of mouse preimplantation embryos (d'Estaing et al, 2001). Only non-essential amino acids are included in the medium for culture to the eight-cell stage followed by all 20 amino acids for culture to the blastocyst stage. Recently Devreker et al. (2001) have shown that supplementing Earle's balanced salt solution with non-essential or 20 amino acids to form two media that were used sequentially to culture human zygotes stimulated the total number of cells in the blastocysts that developed.

To date, the strategies that have been employed to determine the concentrations of each of the amino acids to use in media for the culture of preimplantation embryos do not take account of possible interactions between the effects of these compounds. Thus, it is certain that no currently used media contain optimal concentrations.

SEQUENTIAL CULTURE METHODS

The first use of a stepwise protocol for the culture of mouse preimplantation embryos was described by Chatot et al. (1989) when they described medium CZB. This medium, which contains no glucose, was used to support development to the eight cell/morula stage, at which time glucose is injected into the microdroplet containing the embryos to support further development to the blastocyst stage. Sequential protocols did not receive close attention until a pair of media were introduced, denoted G1 and G2, for the

extended sequential culture of human zygotes into blastocysts (Gardner, 1994; Barnes et al 1995; Gardner and Lane, 1997). Subsequently, Jones et al. (1998) recommended an alternative protocol in which human zygotes were cultured in medium IVF-50 for two or three days followed by culture in medium G2 for a further two to four days. Medium IVF-50 is a commercial medium sold by Scandinavian IVF Sciences AB (now Vitro Life, Gotenburg, Sweden). Unfortunately, for commercial reasons, the composition of this medium is only disclosed qualitatively. The concentrations of each constituent are not made available.

Media G1 and G2 differ in the following six respects: (1) lactate is reduced from 21 to 11.74 mmol/l, (2) pyruvate is reduced from 0.32 to 0.10 mmol/l, (3) glucose is increased from 0.50 to 3.15 mmol/l, (4) taurine is reduced from 0.1 to 0 mmol/l, (5) the seven Eagle's non-essential amino acids only are present in G1 while all 20 natural amino acids are present in G2, and (6) EDTA is reduced from 0.1 to 0 mmol/l. The justification of the first three changes is based on the "back to nature" principle, while the last three changes are based on "let the embryos choose" principle.

One reason for renewing a medium during a period of culture is to remove any toxic substances, such as ammonia, that may have accumulated. The benefit of so-doing in the cultivation of mouse embryos was demonstrated by Gardner and Lane (1993). One objection to renewing the medium is that it removes any putative embryo-derived factors that may be needed for blastocyst development (Gardner, 1994). The loss of these factors can be overcome by the use of dipeptides such as glycylglutamine (Biggers et al., unpublished) or by reducing the amino acid concentration.

Gardner (1998) explains the need for replacing a culture medium with another of different composition at about the eight cell stage of human development, such as G1 with G2, as follows:

“in order to support development of a competent zygote to the blastocyst stage, one needs to use more than one culture medium to take into account the significant changes in embryo physiology and metabolism which occur during the preimplantation period”.

This intuitively reasonable assertion is based on the "back to nature" principle. It is, however, a heuristic argument depending on the truth of two hypotheses. Firstly, the environments of the preimplantation embryo change as they travel from the ampulla of the oviduct to the uterus. As shown earlier, extant evidence that this hypothesis is true is based on weak information from analyses if glucose and pyruvate in oviductal and uterine fluid collected from non-pregnant women (Gardner et al., 1996). Secondly, the compositions of the environments that an embryo encounters have evolved so as to be highly

correlated with the embryo's metabolism. This reasoning is based on the well documented observations in animal models and humans that in early development, the embryo preferentially utilizes pyruvate over glucose and that around the time of compaction this preference is reversed so that the embryo uses glucose rather than pyruvate. These changes in the preferential use of glucose and pyruvate appears complimentary to observed changes in the composition of fluids in the oviduct and uterus during the menstrual cycle. Gardner's explanation lacks rigor by overlooking the possibility that the embryos may adapt so that they can selectively take what they require from a constant environment.

It has been reiterated many times that a two-step culture protocol is superior to a protocol in which a single medium is used throughout without renewal. This claim has resulted in a proliferation of protocols using pairs of commercially available media marketed by several companies (Table 12.4). Many of the companies advertise that the compositions of their paired media optimize the production of blastocysts from zygotes *in vitro*. It is impossible to evaluate critically these claims since precise definitions of what is being optimized are not stated, and, for commercial reasons, only the constituents of these media have been revealed and not their concentrations. Papers are now beginning to be published comparing some of these different commercial protocols (Devreker et al, 2001; Langendonckt et al, 2001). Very few studies have been done asking the more fundamental, practically important, question: What is the evidence that justifies the use of two-step protocols instead of a one-step protocol where the medium is left unchanged for a culture period of five days? Recently Biggers and Racowsky (2002) have shown that blastocysts develop with high efficiency in five days using medium KSOM^{AA} (Biggers et al, 2000) without renewal of the medium. The yield did not differ from the efficiency of a two-step protocol used routinely. Macklon et al. (2002) have also found no differences in the rates of blastocyst formation, implantation and pregnancy in the human using a one-step culture system, using a chemically defined medium supplemented with plasma proteins, and the commercially available G1/G2 sequential two-step culture system. Further work, comparing development using two-step and one-step protocols may show that, although two-step procedures are sufficient to support development, they may not be necessary.

CELLULAR HOMEOSTASIS

Minor stresses are inevitably placed on preimplantation embryos grown *in vitro* in chemically defined media. The stresses arise because chemically defined media provide only a partial representation of the natural environment in which the embryos develop. The fact that embryos will grow under these conditions suggests that they can compensate and/or adapt to the imposed stresses (Biggers, 1993, 1998; Leese, 1995; Lane, 2001). For example, metabolic processes in the embryos continually deplete or add substances to the medium that affect the intercellular pH and cell volume of cells, thereby imposing minor stresses. The embryo counteracts these perturbations so as to maintain the intracellular pH and cell volume at the normal set points of these parameters (for reviews see: Biggers et al, 1991; Lane and Gardner, 2000; Biggers, 2002). If the imposed pH and osmotically-related stresses are too great these responses will fail and the embryos die.

TABLE 12.4. COMPANIES THAT HAVE DESIGNED MEDIA FOR THE TWO-STEP CULTURE OF HUMAN ZYGOTES TO THE BLASTOCYST STAGE. (THIS LIST IS NOT EXHAUSTIVE.)

Company	Web site
Cook IVF	www.cookivf.com/cultsys.htm
FertiCult N.V.	www.fertipro.com/products/ferticul.htm
InVitro Care Inc.	www.invitrocare.com
Irvine Scientific	www.irvinesci.com
IVF Science Scandinavia	www.ivfscience.com
Laboratoire C.C.D.	www.ccd-lab.com/english/products
Medi-Cult	www.medi-cult.com/fertility/products
SAGE BioPharma	www.e-ivf.com

REGULATION OF PH

A stable acid-base status in the cells of an embryo is essential for supporting its development, to maintain the degree of ionization of the component amino acids in the cellular proteins so that they may function normally (Tyler-Jones and Taylor, 1999). Embryos are cultured in a closed system that consists of three compartments: the embryo, the medium and the gas phase. Thus a stable acid-base status of the embryo depends on the properties and exchanges between these three compartments.

The acid-base status of a mixture of solutes in water is determined by the relative activities of hydrogen (H^+) and hydroxide (OH^-) ions. The control of this ratio is complex. In biological fluids, such as arterial blood plasma, it

depends on three factors that act independently: the strong ion difference, the carbon dioxide partial pressure and the total weak acid present (Stewart, 1978). The strong ion difference is the difference in the concentrations of strong cations and anions, the most important of which are sodium, potassium, magnesium, chloride and lactate. In a culture system using a chemically defined medium all three of these factors are held constant, and the solution is buffered using a CO₂/bicarbonate system. Thus, the pH, and hence the [H⁺], can be approximately estimated using the Henderson-Hasselbach equation:

$$\text{pH} = \text{pK}' + \log [\text{HCO}_3]/[\text{CO}_2]. \quad (1)$$

pK' is the pH when [HCO₃] and [CO₂] are equal, which depends on other factors such as ionic strength and temperature.

A practically useful form of this equation is:

$$\text{pH} = 6.1 + \log [[\text{NaHCO}_3]/(0.03 \times \text{PaCO}_2)] \quad (2)$$

where PaCO₂ is the partial pressure of CO₂ in mmHg, [NaHCO₃] is the concentration of NaHCO₃ in mmole/l, and pK'= 6.1. pK'=6.1 is the value when the solution is the ionic strength of blood and the temperature is 37°C (Chang, 2000). For example, if [NaHCO₃] is 25mmole/l, and if the medium is gassed with 5% CO₂ in an atmospheric pressure of 740 mmHg where the PaCO₂ is 740 x 5/100=37 mmHg, the estimated pH is 7.45. These calculations are readily done on the following website:
<http://medcalc3000.com/HendersonHasselbach.htm>.

Figure 12.7 shows the effect of different [NaHCO₃] and [CO₂] on pH using equation 2. There is considerable latitude in the choice of concentrations of these two substances in order to maintain the pH between 7.0 and 7.5. The adoption of 25 mmole/l NaHCO₃ and 5% CO₂, which is widely used for the culture of preimplantation embryos, began with the work of Whitten (1956). The choices were based on the concentration of NaHCO₃ in Krebs-Ringer solution (24.88 mmole/l), described by Krebs and Henseleit (1932), on which Whitten's medium was based. It is important to recognize that the Henderson-Hasselbach equation only approximates the pH that is found in culture media. This fact is illustrated in figure 12. 8 and based on the data of Brinster (1965b) who measured the pH of a simple medium containing 10.15 mmol/l of sodium lactate gassed with 5% CO₂ in which [NaHCO₃] was varied. The Henderson-Hasselbach equation slightly overestimated the pH at all concentrations of [NaHCO₃].

From a physiological point of view, pH is an unsatisfactory parameter to evaluate the acid-base status of a system (Tyler-Jones and Taylor, 1999). It is more realistic to use the actual H^+ concentration, rather than pH which can distort reality since it is a non-linear transformation of $[\text{H}^+]$. There is about 36 nmoles/l $[\text{H}^+]$ ($\text{pH}=7.44$) in a medium containing 25 mmol/l $[\text{NaHCO}_3]$ gassed with 5% CO_2 at 760 mmHg. This value is almost one half the $[\text{H}^+]$ concentration of 63 nmoles/l ($\text{pH}=7.2$) in human embryonic cells (Phillips et al, 2000).

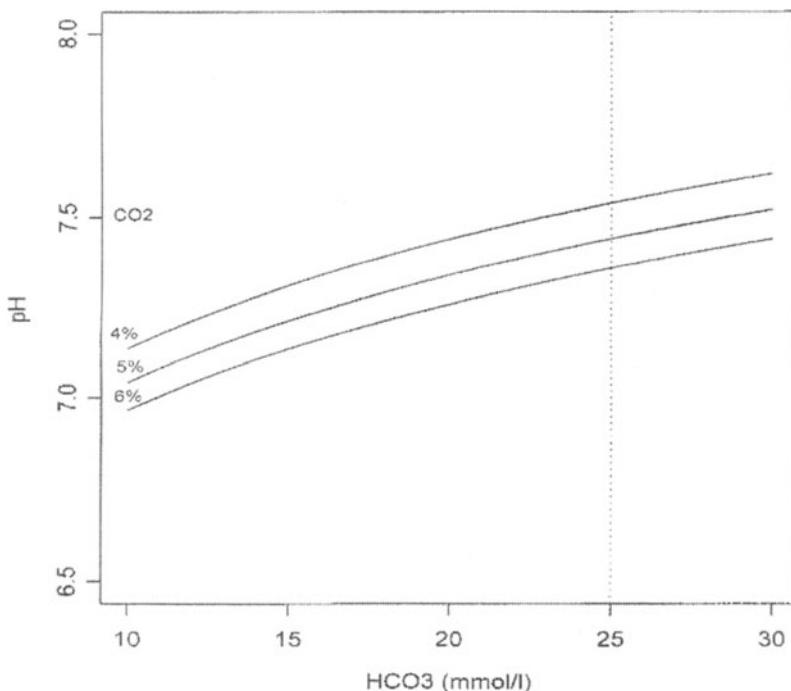


FIGURE 12.7. The effect of varying the concentrations of HCO_3^- and CO_2 on pH, calculated from the Henderson-Hasselbach equation.

Knowledge of the control of intracellular pH in the cells of the preimplantation embryo has increased rapidly in recent years from studies which permute the extracellular environment (for reviews see: Biggers et al, 1991; Kane and Gardner, 2000). The work has been done mainly on mouse embryos supplemented by other studies on hamster and human embryos. The intracellular pH of the cells of preimplantation embryos is controlled locally and rapidly by the intrinsic buffers such as the zwitterionic amino acids. More persistent changes in the intracellular pH are regulated more slowly by the

activities of ionic transport systems in the cell membrane. Studies on the human preimplantation embryo have shown that intracellular alkalosis and acidosis are compensated by transporters analogous to those found in the mouse (Phillips et al, 2000). Recovery from alkalosis is mediated by a Cl^- - HCO_3^- exchanger that is activated when the pH > 7.2. Recovery from acidosis involves two transporters: a tentatively identified Na^+ , Cl - HCO_3^-

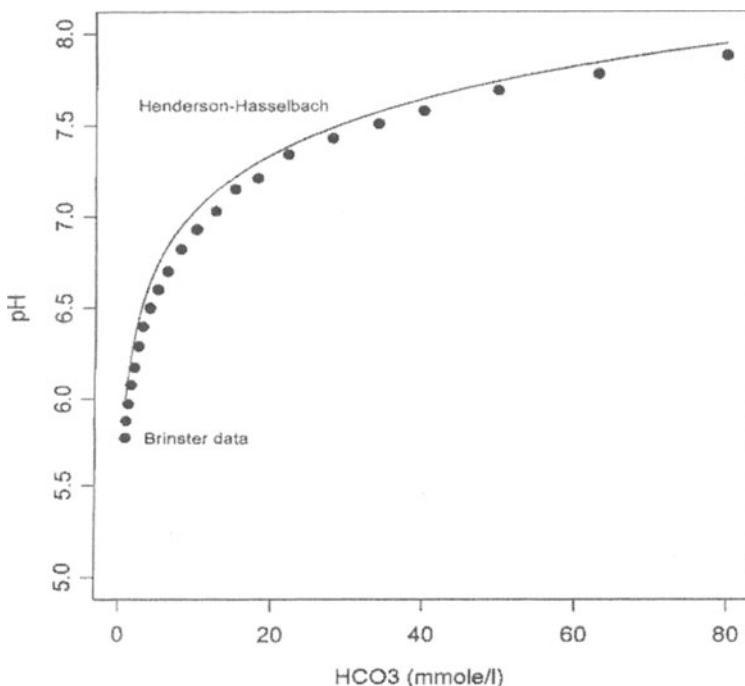


FIGURE 12.8. The relation between (a) the concentration of HCO_3^- under a gas phase of 5% CO_2 and pH calculated using the Henderson-Hasselbach equation, and (b) the experimental measurement by Brinster (1965b) of the pH of a base medium containing different concentrations of HCO_3^- under a gas phase of 5% CO_2 .

exchanger which is activated when the pH < 7.0 and a Na^+ - H^+ antiporter which is activated when the pH < 6.8. These three transporters collectively maintain the pH of blastomeres at set point that falls between 7.0 and 7.3, provided HCO_3^- and CO_2 are present.

Brinster (1965b) showed that mouse embryos can develop over a wide range of pH (5.87-7.78). The implication of this result is clarified by plotting the results of the percent development against the $[\text{H}^+]$ (Figure 12. 9). The

results show that mouse embryos can develop in a range of $[H^+]$ ranging from 16.6 nmol/l (pH 7.78) to 1349 nmol/l (pH 5.87) with a maximum at 151 nmol/l (pH 6.82). The concentration-response line is not symmetrical being much steeper on the left of the maximum than the right. Thus, relatively small reductions in $[H^+]$ from the optimum $[H^+]$ have a pronounced inhibitory effect on development. In contrast, comparable increases in $[H^+]$ above the optimum $[H^+]$ have relatively little inhibitory effect. There is considerable evidence from several species that the pH of oviductal fluid is alkaline in the range pH 7.7-8.2. Thus, the Cl^- - HCO_3^- exchanger in the blastomeres of the early embryo developing *in vivo* would be continually active. Buffering a medium to maintain a pH~7.2 using a HCO_3^- /CO₂ system would remove the naturally occurring stimulation of the Cl^- - HCO_3^- exchanger.

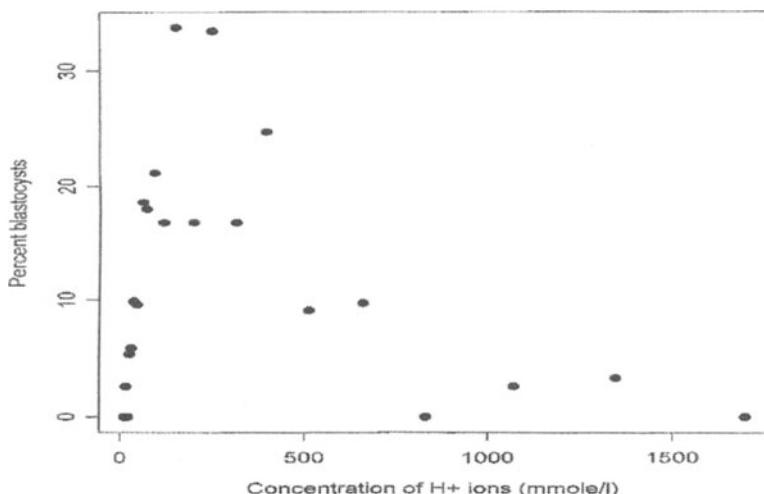


FIGURE 12.9. The development of two cell mouse embryos into blastocysts *in vitro* in a base medium containing different concentrations of H⁺. (calculated from the data of Brinster (1965b).

REGULATION OF CELL VOLUME

The effects of varying the tonicity of a medium surrounding cells has been traditionally explained by the assumption that the cell membrane is a semi-permeable membrane. It is now known that delicately balanced mechanisms are brought into play to maintain a normal cell volume after cells are exposed either to hypotonic or hypertonic solutions (for review see: Lang et al, 1998; Lange, 2000). Three responses may occur in sequence when a cell is placed in

a hypertonic solution (12. 10). The initial response is rapid and involves the movement of water out of the cell causing it to shrink. This reduction in volume stimulates a second slower response in which ions and/or organic osmolytes move from the environment into the cell, thereby restoring the cell volume. If the cell volume is still not restored, a very slow third response occurs in which genes are activated to stimulate the synthesis of organic osmolytes intracellularly. When cells are exposed to hypotonic solutions the reverse of these processes occur.

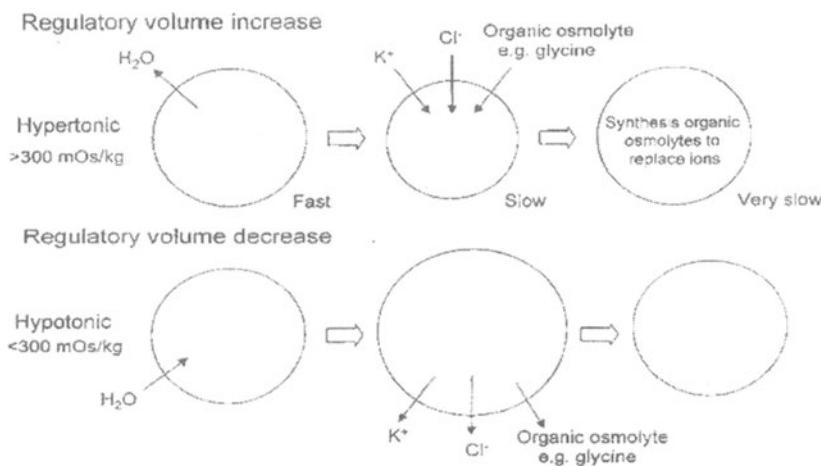


FIGURE 12.10. The control of regulatory volume increase and regulatory volume decrease in response to hypertonicity and hypotonicity. (from Biggers, 2002).

Considerable advances have been made in recent years in our understanding of the regulation of cell volume in the preimplantation embryo of the mouse (for review see: Baltz, 2001). The effect of varying the concentration of NaCl in a medium on the development of a mouse zygote into a blastocyst *in vitro* is shown in Figure 12.11. The development of the embryos is severely impaired when the NaCl concentration exceeds ~115 mmoles/l (290 mOsM), but is not impaired if the NaCl concentration is as low as 80 mmol/l (~230 mOsm). The addition of an organic osmolyte, glutamine, to the medium significantly protects the embryo from the injurious effects of high concentrations of NaCl. Several other compounds have been shown to act as organic osmolytes in mouse preimplantation embryos: glycine, betaine,

proline, alanine and hypotaurine (Van Winkle et al, 1990; Biggers et al, 1993; Dawson and Baltz, 1997). The latest evidence demonstrates that the cell volume of preimplantation embryos is regulated by three transporters, located in the cell membrane, molecules which function as organic osmolytes. These transporters are GLY which imports glycine, glutamine and other compounds into the cell, beta (β) which imports taurine, alanine and other amino acids, and VSOAC which exports organic osmolytes (Figure 12.10). The joint action of these transporters, which are sensitive to changes in cell volume, maintains the intracellular ionic strength at levels that do not impair cellular functions and embryonic development in the face of high external osmolarity. Also, the activity of GLY and VSOAC falls while the activity of β rises with development. It is not known whether genes present in preimplantation embryos exposed over a prolonged time to hypertonic solutions in the absence of organic osmolytes can be activated to synthesize organic osmolytes.

That the osmolarity of oviductal fluid is high has been known for many years. The latest estimate in the mouse is ~300 mOsm (Collins and Baltz, 1999). Paradoxically, the osmolarity of many effective media for the culture of preimplantation embryos is significantly lower, 250-270 mOsm. The suggestion has been made that preimplantation embryos, particularly the zygote, maintain an intracellular ionic strength sufficient to offset an external tonicity of 250-270 mOsm (Baltz, 2001). If the embryos are in an environment with higher tonicity, as *in vivo* or in media with higher tonicity, the balance is made up by importing organic osmolytes. Thus the beneficial effects of adding certain amino acids to media for the culture of preimplantation embryos may be due to their potential roles as organic osmolytes. In the design of media it is clearly wrong to add inorganic salts to a medium to raise its osmolarity to that present in oviductal fluid.

PATHOPHYSIOLOGY ARISING FROM STRESSES DUE TO CULTURE

There is increasing interest in whether the conditions used for the culture of preimplantation embryos have long-term deleterious effects. Stresses occur when homeostatic mechanisms are overcome and under extreme conditions the embryos die. Of particular interest are those conditions where the embryos survive but may be seriously impaired. For development, concerns range from short-term effects such as generation of high concentrations of free radicals, particularly reactive oxygen species (Johnson and Nasr-Esfahani, 1993) to long-term effects that are only manifested later in development after the cultured embryos are transferred to surrogate mothers (Rieger, 1998; Khosla et al, 2001). Many of these topics fall outside the scope of this review. Three areas, however, are briefly summarized here: the effects of reactive oxygen

species, the abnormal activation of genes, and some general aspects of the cellular response to stress.

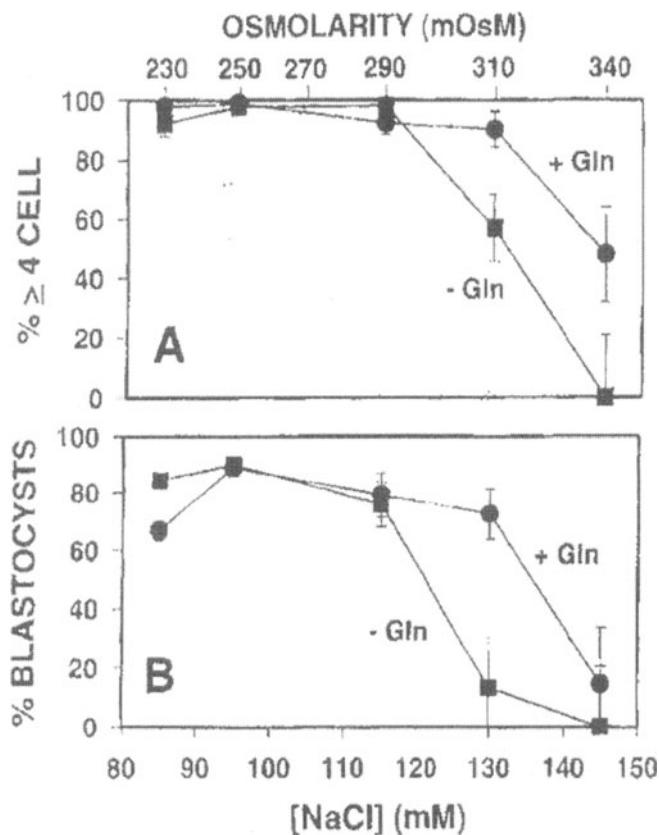


FIGURE 12.11. The development of mouse zygotes in KSOM in media of different osmolarity produced by varying the concentration of NaCl with and without glutamine. (from Dawson and Baltz, 1997).

REACTIVE OXYGEN SPECIES AND PREIMPLANTATION DEVELOPMENT IN VITRO

Under certain conditions dioxygen (O_2) can give rise to the reactive oxygen species: superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl ion (OH^{\cdot}) (Cadenas and Davies, 2000). OH^{\cdot} , the most reactive of these species that can damage DNA, RNA and proteins, is produced when

homeostatic mechanisms that maintain the balance between O_2^* and H_2O_2 are insufficient. O_2^* can also react with nitric oxide (NO^*), a reactive nitrogen species, to produce peroxy nitrite ($ONOO^-$) which can damage lipids, proteins and DNA (Ghafoorifar and Richter, 1997; Vinent-Johansen, 2000). Reactive oxygen species are produced by preimplantation mouse embryos only in culture and only at the G₂/M stage of the second cell cycle (Nasr-Esfahani and Johnson, 1990). Also, the embryo is sensitive to NO^* only at the mid- to late 2-cell stage of development (Warren et al, 1990).

In pioneer work on the culture of mouse preimplantation embryos, culture droplets were gassed with 5% CO₂ in air which contains about 20% O₂ (Whitten, 1956; Brinster, 1963). Later, Whitten (1970) claimed that mouse zygotes would not cleave unless the cultures were gassed with 5% CO₂, 5% O₂ and 90% N₂. Over the years, considerable variation occurred between different laboratories as to which of these two gassing mixtures should be used. Since the oxygen tension in the mammalian oviduct is low, in the range of 1-9% (Fischer and Bavister, 1993), it has been argued that a gas mixture with a lower oxygen tension is preferable. Using an oxygen tension above normal could favor the production of excess reactive oxygen species. Possibly the minimization of the production of reactive oxygen species explains why Orsi and Leese (2001) have recently shown that gassing a medium with 5%, rather than 20% O₂, for the culture of preimplantation mouse embryos results in a higher yield of blastocysts with larger cell numbers. Similar studies on the human preimplantation embryo have not shown any significant advantage of a low oxygen tension on development over the first two to three days of development *in vitro* (Noda et al, 1994; Dumoulin et al, 1995, 1999). Although the effect of oxygen tension on preimplantation development is still not clear, it seems that the use of a low oxygen tension for the gassing of culture media is preferable.

GENE EXPRESSION

There is ample evidence that genes control the development of preimplantation embryos (for review see: Warner and Brenner, 2001). The advent of ultra-sensitive methods has demonstrated the expression of a large number of active genes during the preimplantation period of development. (Doi, 2000; Latham et al, 2000). After mapping 798 genes in the mouse it was concluded that “(i) a significant fraction of the genome is dedicated to genes expressed specifically in early development,... (ii) genes co-expressed in the same stage tend to cluster in the genome, and (iii) the expressed genes include cohorts acting in a stage-specific manner...” (Latham et al, 2000). Thus, there is considerable opportunity for the disruption of the activity of these genes

when embryos are removed from their natural environment and manipulated *in vitro* (for review see: Niemann and Wrenzycki, 2000).

Three types of information have accumulated on the effects of culture on gene activity in the mouse. Firstly, there are comparisons of the activities of genes in either embryos developing *in vivo* with those developing *in vitro*. Shim et al. (1996) found that the development of laminin chain-specific mRNA transcripts was less in embryos developing in medium M16 (Whittingham, 1971) than those developing *in vivo*. Similarly, Uechi et al. (1997) estimated that the level of expression of the glucose transporter GLUT-1 in 2-cell embryos grown in medium BWW (Biggers et al, 1971) was one tenth of that in embryos that developed *in vivo*. The observation of an inhibitory effect of culture conditions compared with natural development *in vivo* should always be scrutinized carefully, as the effect may be due to a generalized slowing down of development arising from stressful culture conditions. Fleming et al. (1997) found no difference in the appearance of some members of the growth arrest genes (gas and gadd family) in preimplantation embryos that developed *in vivo* compared with those cultured in medium KSOM (Lawitts and Biggers, 1993). Christians et al. (1995) reported a significant increase in the HSP 70.1 gene in embryos cultured in medium M16 compared with the increase that occurs *in vivo* (Figure 12.12).

Secondly, comparisons have been made on the expression of genes in embryos cultured in different media. Ho et al. (1995) reported that the expression of mRNA of some genes (actin, G3PDH, Na⁺/K⁺-ATPase, Sp1, Tata box binding protein) were the same when cultured in Whitten's medium (Whitten, 1970) and medium KSOM supplemented with half strength Eagle's amino acids (KSOM^{AA}). In contrast, four genes (IGF-I, IGF-IR, IGF-II, IGF-II) were significantly higher in the embryos cultured in KSOM^{AA} than those in Whitten's medium. Doherty et al. (2000) have also examined the expression of the H19 gene, concerned with imprinting, in embryos cultured in Whitten's medium and KSOM^{AA}. It was found that the gene was expressed normally by only the maternal allele in embryos cultured in KSOM^{AA} but abnormally in embryos cultured in Whitten's medium where it was expressed in both the maternal and paternal alleles.

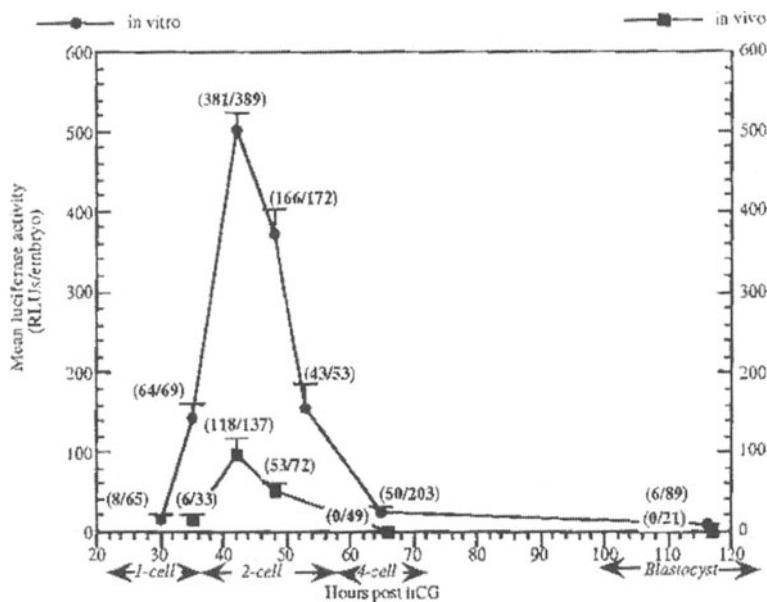


FIGURE 12.12. Expression of HSP70, measured by luciferase activity, at different stages of mouse development *in vivo* and *in vitro*. (from Christians et al., 1995).

Thirdly, comparisons have been made of the expression of genes in embryos cultured in the same medium in which the concentration of a constituent is measured. Ho et al. (1995) showed that the expression of mRNAs of IGF-I, IGF-IR, IGF-II and IGF-IIR in embryos cultured in medium SOM (Lawitts and Bigger, 1993) was reduced when the concentration of NaCl was increased from 85-125 mmol/l.

The results of studies on the mouse demonstrate that the composition of chemically defined media can significantly effect the expression of genes in the preimplantation embryo. No information is available on the effect of culture media on the expression of genes in the development of the human preimplantation embryo but undoubtedly the expression of genes could be effected and is of concern.

OTHER ASPECTS OF STRESS

The subject dealing with the effects of stress on cells at the molecular level is a particularly active area of research at the present time. Substances such as

the heat shock proteins, CHOP-10 and ceramide are involved. For example, inhibiting the heat shock protein Hsp70 by its complimentary antisense oligonucleotide sensitizes the four cell mouse embryo to arsenite (Dix et al, 1998; Luft and Dix, 1999). The gene CHOP-10 is overexpressed in mouse and bovine blastocysts exposed to stresses such as arsenite or the alkylating agent methyl methane-supphonate (Fontainier-Razzaq et al, 1999,2001). Ceramide, a compound involved in sphingolipid metabolism, increases in cells exposed to stress (for reviews see: Mathias et al, 1998; Ronai, 1999; Hannun and Luberto, 2000), but so far this compound has not been studied in the preimplantation embryo. CHOP-10 and ceramide have been shown to be involved in the control of cell cycle arrest and apoptosis. Could it be that these substances are produced by stresses induced by unbalanced chemically defined media and that they potentially effect the later development of the fetus?

CONCLUDING COMMENTS

Hardy et al. (2001) proposed that the limiting factor controlling the efficiency of human IVF is the health of the zygote. They suggested that research should be focused on the problem of improving the quality of the zygote produced by IVF, rather than on media development. The justification of improving media, however, involves not only improving the yield of embryos for transfer but also to minimize the effects of stress which may impair later fetal development. From the early work of Edwards et al (1981) it has been known that human preimplantation embryos can develop in media of very different composition. It is now clear, particularly from studies of non-human species, that some media are better than others. These are media that allow development of the zygote to the blastocyst stage with adequate numbers of inner mass and trophectoderm cells together with their supporting extracellular matrix. The improvements are due in part to including key constituents, but also the choice of the concentrations of each the constituents that support growth and development and also minimize the inherent effects of stress inevitably imposed by chemically defined media. Further, it is imperative that the concentrations of the constituents be determined when they act in concert. Since knowledge of these concentrations is so important, it is particularly disturbing that the quantitative concentrations of the majority of commercially available media are unavailable for protective commercial reasons (Biggers, 2000). The claims of the manufacturers of the superior quality of their products can only be ascertained by comparing their effects one with another. Unless the concentrations of the components are known the media cannot be subject to critical scientific analysis. Greater progress could

be made in the treatment of human infertility if the composition of all media is disclosed.

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CHAPTER THIRTEEN

THE BIOLOGICAL BASIS OF OOCYTE AND EMBRYO COMPETENCE: MORPHODYNAMIC CRITERIA FOR EMBRYO SELECTION IN IN - VITRO FERTILIZATION

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INTRODUCTION

The implantation rate of embryos in human assisted reproduction (ART) is low with between 10 and 40% of transferred embryos implanting. It has been estimated that the overall natural fecundity in normal healthy couples is approximately 20-25% in any one cycle, not too different from that seen in ART, however the expectation of doing better than nature has crept into the practice of ART (Edwards and Beard, 1997). To increase success rates, some practitioners have resorted to multiple embryo replacements with a resulting escalation of high-order pregnancies worldwide without a corresponding increase in the overall implantation rate (Racowsky, 2002). Some countries have mandated limits to the number of embryos that can be replaced (Ludwig et al., 2000) and others have proposed guidelines and recommendations to reduce the risk of higher order gestations. To comply with the regulations and limit the number of embryos replaced while not reducing the probability of implantation requires reliable methods of selecting embryos with the most potential for implantation. If it is assumed that human embryos obtained by in vitro fertilization (IVF) at different ART centers have after similar ranges of competence after fertilization, a 4-fold difference in implantation rate is troublesome and suggests that clinic-specific practices and procedures have an important impact on outcome. This chapter reviews the relevant findings pertaining to how signs of high competence can be recognized at the earliest stages of human embryo development in vitro and describes criteria that, if adopted by IVF programs, should significantly improve outcome in centers where ongoing pregnancy rates persist at low levels.

Since the initiation of ART as a clinical practice (Edwards et al., 1980), many improvements have been made in all aspects of the science. One of the leading contributions to the embryology laboratory has been an improved understanding of the conditions required for the in vitro culture of gametes and embryos. Over the last 50-60 years animal research has led to the development of media that are consistent with full development of embryos through the preimplantation stages to the hatched blastocyst. These have included the development of fertilization media and a more detailed understanding the dynamic interaction between metabolites, amino acids, inorganic phosphate and salts which appears to be required to support embryogenesis through the preimplantation stages (see Biggers, this volume). Many of the findings from animal models have been rapidly incorporated into clinical IVF, often with little relevant experimentation or clear validation. At present there are a wide variety of culture systems in use for human IVF (Rawlins, 2001). All current media are based primarily on simple balanced salt solutions, such as Earles Balanced salt solution or Tyrodes medium, although some media are relatively complex and are derivatives of media used for tissue culture, such as Ham's F10. While both simple and complex media are largely equivalent in their ability to support the early development of the human embryo in vitro and, when embryos are transferred at the cleavage stages result in similar ongoing pregnancy rates (Scott, 2001a), differences have been observed when culture is extended to day 5 or 6 in order to permit blastocyst formation (Alvero et al., 2000). Failed implantation after the transfer of stage-appropriate cleavage stage embryos (and blastocysts as well) and the inability of some proportion of embryos within and between cohorts to develop to the blastocyst stage in vitro illustrate a fundamental principal of early human embryogenesis, namely, that it cannot be assumed *a priori* that mature oocytes which appear equivalent at the light microscopic level have equivalent developmental potentials or if fertilized, are equally capable of producing competent embryos that can develop to term. While human embryo culture media have yet to be truly 'optimized,' present formulations have enabled embryologists to repeatedly observe human embryos during culture and to develop selection strategies that lead to increased pregnancy rates in environments where the number of transferred embryos is limited by protocol or legislation.

CURRENT EMBRYO SELECTION STRATEGIES

Morphology is the most common method of selecting embryos for transfer because it is obviously the simplest. There are multiple scoring systems for each stage of development with corresponding clinical outcome data reporting correlations with implantation. The essential question is to what extent do

morphological assessments correspond to competence and therefore as a selective mechanism, what is its predictive of outcome. Morphological indications of competence have been used to select embryos at the 1-cell (day 1: pronuclear) (Balaban et al., 2001; Ludwig et al., 2000; Payne et al., 1997; Scott et al., 2000; Scott and Smith, 1998; Tesarik and Greco, 1999; Wittemer et al., 2000) 2-cell (Bos-Mikich et al., 2001; Edwards et al., 1980; Lundin et al., 2001; Petersen et al., 2001; Sakkas et al., 2002; Shoukir et al., 1997) and 8-cell stages (Dawson et al., 1995; Desai et al., 2000; Puissant et al., 1987; Rijnders and Jansen, 1998; Steer et al., 1992; Tan et al., 1992; Van Royen et al., 2001). Morphological criteria indicative of high competence used for selection during the cleavage stages include the uniformity of cell division (Hardarson et al., 2001; Roux et al., 1995), degree and pattern of fragmentation (Alikani et al., 2000; Antczak and Van Blerkom, 1999; Desai et al., 2000; Van Blerkom et al., 2001) and the rate of development (Racowsky et al., 2000; Shapiro et al., 2000). The introduction of extended culture media for blastocyst development has allowed further selection of human embryos by two methods: (1) attrition, in which some fraction of embryos simply arrest development and do not develop to the blastocyst stage and (2) blastocyst morphology as determined by the presence of a stage appropriate inner cell mass (ICM) and trophectoderm, both of which have been suggested to contribute to increased pregnancy rates with one or two transferred embryos (Dokras et al., 1993; Gardner and Lane, 1997; Gardner, et al., 1998; Gianaroli et al., 2000; Scholtes and Zeilmaker, 1998). However, for some countries where the number of oocytes that can be inseminated is proscribed by law, or where cryopreservation or embryo culture beyond the pronuclear/early cleavage stages is prohibited, selection strategies beyond day 1 or 2 are not viable options.

In clinical practice, the real value of any scoring system is often difficult to establish unambiguously because the imperative to have a positive outcome in an IVF cycle often results in groups of embryos with different ‘scores’ being transferred, thereby making correlations between a specific morphology and outcome problematic. Further, it is common experience that even when two or three embryos with the same score are replaced only 1 implants, indicating that differences other than morphological exist between embryos. This clearly presents a problem to the embryologist attempting to select the most viable embryos for transfer or cryopreservation, for in most IVF laboratories; the single most important and available tool is observation. Building a ‘competence profile’, each stage leading to the next, should enable the choice of embryos by sequential selection/elimination. However, any one-observation only provides a “snap shot” in time and may not be a true indication of either an embryos progress or potential. Observation and scoring must be done in a timely fashion at points that are meaningful for embryo

assessment (Table 13.1). What is observed needs to be understood and interpreted in a biological context; that is, what development processes or events that are indicative of normal human embryogenesis have morphological correlates and therefore provide meaningful developmental landmarks? This chapter is intended to provide the ‘biological context’ for morphology scoring and discusses critical aspects of oogenesis and early embryogenesis that are necessary for the establishment of developmental competence.

To have normal embryogenesis requires that the gametes from which the embryo arises are both normal and competent. Abnormal gametes are unlikely to be rescued *in vitro*, implying that the development of developmental competence of the embryo begins in the oocyte. Although the oocyte within the follicle cannot be scored, understanding the events that lead to a competent oocyte will help in selecting embryos that do have potential.

TABLE 13.1. FLOW OF SEQUENTIAL SCORING CURRENTLY AVAILABLE TO THE ART LABORATORY FOR EMBRYO SELECTION

STAGE OF SCORING	PARAMETER
Follicular Development	E2 rise, follicle growth
Oocyte retrieval	Blood flow to ovary ATP levels in follicular fluid
Metaphase II oocyte	Oocyte maturity Morphology of first Polar Body Metaphase spindle integrity Metaphase spindle position
Pronuclear Embryo	Pronuclei position Nucleoli morphology Cytoplasmic halo Early cleavage
2-Cell Embryo	Morphology Multinucleation
4-8-cell embryo	Fragmentation Multinucleation Blastomere size and shape Timing of development
Blastocysts	Timing of formation Evenness of trophectoderm Distinct ICM Blastocoel

OOCYTE DEVELOPMENT

In order to understand the extent to which competence is established in the oocyte, the normal molecular and cellular events, which characterize oogenesis, need to be understood. While the following discussion is not intended to be a comprehensive discussion of mammalian oogenesis, selected events in this process, which can contribute to developmental competence, both for the human oocyte and early embryo are described.

Oogenesis and the development of a fully-grown, fertilizable human gamete occur over a period of months (see Cavilla and Hartshorne, this volume). As in other mammals, primordial human follicles are triggered to develop by signals that remain to be clearly identified, but once development is initiated, growth is progressive to the preantral stage. The oocyte within these developing follicles is surrounded by three layers of granulosa cells but they are not competent to resume meiosis (Sorenson and Wassarman, 1976). Human oocyte growth is also progressive, increasing in size about 300 times to a fully grown diameter of approximately 100 microns, and it is during this phase the zona pellucida is laid down and the somatic cells of the follicle, the granulosa cells, proliferate. At the terminal stages of the growth phase, fluid accumulates within spaces between granulosa cells forming an early cavity or antrum. Mature ovaries contain pools of fully-grown oocytes arrested in prophase of the first meiotic division, which are in a dormant or resting phase with no RNA synthesis. At this stage, oocytes are capable of responding to gonadotropins by resuming meiosis (Eppig, 1990,2001). The ability to resume meiosis is accompanied by further development involving an increase in the size of the oocyte nucleus (germinal vesicle), which alters the nuclear to cytoplasmic ratio, and the resumption of ribosomal RNA synthesis (Crozet et al., 1986).

The final phases of folliculogenesis and oogenesis involves a highly coordinated series of molecular and cellular events and processes culminating in the preovulatory nuclear and cytoplasmic maturation of the oocyte and growth and differentiation of the granulosa cells (Albertini et al., 2001; Eppig, 2001). During oocyte maturation RNA synthesis decreases and the single nucleolus present in the GV stage oocyte disappears. The nucleolus is broken down into its formative parts (see nucleolar structure) and these are scattered through the nucleus (Crozet et al., 1986) and no RNA synthesis occurs. Finally resumption of the first meiotic division and progression to metaphase II occurs prior to ovulation.

An oocyte granulosa cell regulatory loop operates within the ovary and is essential for both oocyte and follicle development (Eppig, 2001). As the

oocyte grows the cells around it grow in a coordinated manner with synchronous development and a high level of communication between the oocyte and its companion somatic cells by means of gap junctions (see Albertini this volume). The somatic cells provide a flow of nutrients to the oocyte as well as other factors such as those that maintain the oocyte in meiotic arrest, such as cyclic AMP. The oocyte communicates with the follicle driving steroidogenesis, somatic cell proliferation, differentiation and ultimately cumulus expansion. An example of this highly coordinated loop is the manner in which the oocyte and granulosa cells interact to bring about differentiation and the expression of LH receptors (LHR). The differentiation of the mural granulosa cells and the expression of LHR, which are essential for ovulation, are driven by the oocyte (Eppig, 2001). The granulosa cells also aid in maintaining the oocyte in meiotic arrest (Downs et al., 1986). The granulosa cells produce KIT Ligand (KL), which promotes the survival and development of the oocyte. KL expression in mural granulosa cells is influenced by signals originating from the basal granulosa cells, which are adjacent to the thecal cells. Once fully grown, the oocyte secretes a factor, which suppresses the expression of LHR and KL mRNA since it has reached its full size and does not need to grow anymore (Eppig, 2001).

The oocyte plays an active role in ovulation through promotion of cumulus mass expansion and prostaglandin production, both of which are essential for ovulation (Hess et al., 1999; Eppig, 2001). Without the oocyte the granulosa cells cannot transform into cumulus cells and the cells of the cumulus-oophorus complex play a central role in the resumption of meiosis (Eppig, 2001; Tanghe et al., 2002) and progression beyond meiosis 1 (Shimada and Terada, 2001). The oocyte also influences the expression of many other factors, which are important for follicle development (Albertini et al., 2001; Eppig, 2001). The granulosa cells influence oocyte maturation by means of estrogen (E2) production, without which the final maturation of the oocyte cytoplasm cannot occur. The role of the oocyte-granulosa cell loop is essential for normal development of the oocyte and follicle (Albertini et al., 2001; Eppig, 2001).

Monitoring follicle growth and E2 levels will give an indication of follicular maturation that should also be coupled to oocyte growth and maturation, if the granulosa cell-oocyte loop is in place. Although oocyte maturation does not presume developmental competence, lack of maturation will result in oocytes that have little developmental ability. However, over two decades of experience from ultrasonographic monitoring of follicles and determinations of corresponding E2 levels for human IVF indicates that for any one follicle, these parameters are not necessarily predictive of oocyte maturation and therefore potential competence. During ART cycles and hyperstimulation with exogenous gonadotrophin, the events of follicle and

oocyte maturation can be uncoupled (Hartshorne et al., 1999), unlike in the mouse where the events are absolutely linked (Albertini et al., 2001; Eppig, 2001). This is seen as adequate E2 rise and follicular growth, indicating a fully grown follicle, but the retrieval of immature oocytes that do not develop or mature in vitro, or are clearly arrested in early development (GV phase). Thus the parameters used to measure oocyte growth in the follicle are very inexact, relate to follicle and not oocyte growth and are not predictive of the developmental competence of the retrieved oocyte.

The clinical literature abounds with papers showing the effects on pregnancy rates if one or many parts of granulosa-cell oocyte loop this loop upset during superovulation (Van Blerkom, 1990). If any part of the oocyte-follicle development is perturbed it could have a profound effect on the quality of the resulting oocyte. In vitro maturation of oocytes has highlighted the importance of this loop, where in both bovine (Van Blerkom et al., 1990; Hyttel et al., 2000) and humans, reduced developmental ability of the embryo results. Subtle perturbations in normal intercellular communication and biosynthetic coordination in the follicle/oocyte loop described above may have profound downstream effects, seen later as failure to develop through the preimplantation stages or implant after transfer. One possible example of reduced competence associated with this loop may be associated with reduced perifollicular blood flow as measured by pulsed color Doppler ultrasound (Nargund et al., 1996; Chui et al., 1997; Van Blerkom et al., 1997; Huey et al., 1999; Gregory, this volume). In these studies, oocytes originating from follicles with reduced blood flow had reduced potential, even if the oocytes did fertilize and appeared to develop normally. Lack of adequate blood flow to the follicle could decrease the oxygen being delivered to the follicle, alter the metabolism of the granulosa cells which could effect the oocyte by disrupting the loop. Earlier studies had shown that oocytes from cohorts in which most had relatively low net cytoplasmic ATP were fertilizable and could develop through the cleavage stages, but transferred embryos from these cohorts had high rates of implantation failure (Van Blerkom, et al., 1995). This could result from altered metabolism due to reduced blood flow, again disrupting the oocyte granulosa cell loop. Thus, the status of the follicle and the oocyte can effect the development of the embryo, and that damage that the oocyte incurs may not necessarily be seen at the morphological level.

The fully-grown oocyte, which is arrested at prophase of the first meiotic division, resumes meiosis with activation of the M phase promoting factor (MPF) (Masui and Markert, 1971). MPF function during meiosis is regulated by *c-mos* (cellular viral *mos* oncogene). *C-mos* encodes a serine-threonine protein kinase, which is expressed at high levels during meiotic maturation (Sagat et al., 1988). This has been shown in *Xenopus* (Sagat et al., 1988), mouse (Goldman et al., 1987) and humans, where it is expressed in oocytes

and early embryos (Pal et al., 1994; Heikinheimo et al., 1995) and in meiosis in human oocytes (Hashiba et al., 2001). Protein kinases control most signaling events that need to occur rapidly, in sequence and in a cell system that is actively dividing or preparing to differentiate in some way, as the oocyte does when it resumes meiosis. The events of resumption of meiosis are driven by machinery/systems already in the oocyte, laid down during oogenesis. All these systems that are driven by protein kinases occur too rapidly for the synthesis of new message and always rely on preformed components and just require a trigger (Nasmyth, 1996). In the oocyte MPF is activated when cAMP levels are depleted through the retraction of micro-processes from the coronal cells, which supply the oocyte with cAMP and maintain the oocyte in meiotic arrest. These signals come from the surrounding granulosa cells, which in turn respond to the LH surge. Again, this part of the oocyte-granulosa cell loop and if any part is disrupted the signals could be disrupted, and have profound effects on the competence of the oocyte. Another system that is operational in oocytes is that of cell cycle checkpoints, which are surveillance-like systems that determine which and when cell cycle transitions can occur (Hartwell and Weinert, 1989). Arrest or incorrect completion of a cell cycle event is detected by these surveillance mechanisms and the cell cycle inhibited through inhibitors, thus preventing incorrect completion of the cell cycle (Nasmyth, 1996). Again, if there are disruptions in the oocyte-granulosa cell loop, signals are not transmitted correctly or on time to the oocyte, these check points could prevent the oocyte making the necessary transitions to complete meiosis, leading to an incompetent oocyte.

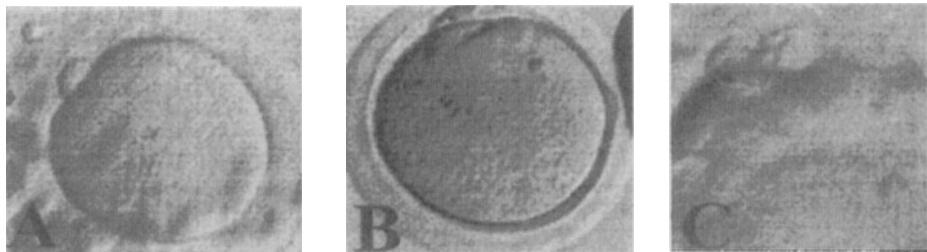


FIGURE 13.1. First Polar Body Morphology at 40 h post-hCG: a: normal, intact polar body ; b: abnormal polar body, beginning to disintegrate, has divided into 2. C: abnormal polar body which is disintegrating and fragmenting.

MEIOSIS I AND THE FIRST POLAR BODY

After the Germinal vesicle breakdown stage of resumed meiosis, the spindle begins to form through the migration of microtubule organizing

centers (MTOCs) to the area where the germinal vesicle was, near the condensing chromosomes (Longo and Chen, 1985; Van Blerkom and Bell, 1986; Verlhac et al., 2000). During this migration and condensation phase crossing-over occurs and this is the most likely stage at which non-disjunction or failure of the sister chromatids to separate, occurs, leading to aneuploidies (Robinson et al., 2001). Once the spindle has formed, its long axis defines the path which the spindle will follow as it migrates to the periphery of the oocyte, which is essential for continued development. This path tends to be the shortest distance to the oolemma. In the human oocyte the spindle does not rotate and is at right angles to the oolemma (Verlhac et al., 2000).

At the completion of meiosis I, there is a halving of DNA content of the oocyte, accomplished through the first reduction division. This results in a small haploid cell, known as the first polar body and a haploid oocyte. The division is highly asymmetric, resulting in a large oocyte and small polar body. This is also controlled by *c-mos* and a mitogen-activated protein kinase, signal transduction pathway (MAPK) (Choi et al., 1996; Verlhac et al., 2000).

The spindle is located directly under the cortex of the oocyte such that very little cytoplasm is eliminated from the cell when the polar body is abstricted. If too much cytoplasm is lost into the polar body, resulting in a large polar body, division has occurred abnormally. This most likely, results from aberrant spindle formation and positioning of the spindle other than on the periphery of the cell, which could result in too much cytoplasm being lost. It has been shown using *c-mos*-knock-out mice, that spindle formation and normal chromosome condensation on the spindles, are dictated by the *c-mos*-MAPK pathway (Araki et al., 1996). The subsequent degradation of the first polar body is also controlled by this pathway (Choi et al., 1996; Verlhac et al., 2000). This degradation is a natural phenomenon, is time dependent, and can give an indication of the extent to which the oocyte is mature, or progressed beyond the stage at which an oocyte would be considered competent due to a time-dependent displacement or deterioration of the spindle. Prior to degradation the polar body contains a full complement of chromosomes, which will rarely contribute to the development of the embryo. This complement is normal as has been demonstrated by their use in nuclear transfer experiments in mice where, prior to the onset of the degradation process, the polar body could fully reconstitute enucleated oocytes with subsequent live births after ICSI (Wakayama and Yanagimachi, 1998). Full degradation is complete by 7 hours in the mouse (Choi et al., 1996) and by about 20 hours in the human (Ortiz et al., 1983). The polar body can divide and fragment in the progress to complete degradation, as seen over time. However, at the time of extrusion, the time at which oocyte competence for ICSI/IVF is evaluated (38-44 h post-hCG) the polar body should still be intact. Polar body morphology could be useful, first-line selection criteria in

ICSI cases, where the granulosa cells are removed allowing full assessment of the polar body (See Fig. 13.1).

METAPHASE II SPINDLE

At ovulation, the oocyte is arrested in the second meiotic division at metaphase II and this arrest is controlled in part by *c-mos*, MAPK and M-Phase promoting factor (MPF) (Murray et al., 1989). The metaphase II spindle is normally bi-polar with microtubules running pole to pole with the chromosomes on the equatorial plane. There are two classes of microtubules in the spindle, long microtubules, which run pole to pole and pass through the metaphase plate, and short microtubules, which are associated with the chromosomes through the kinetochores. The microtubules are not static and turn over rapidly with constant assembly and disassembly of the tubulin molecules (Gorbsky et al., 1990). Cell cycle checkpoints are very important in determining the alignment of the microtubules and the attachment of the chromosomal kinetochores to the spindle (Gorbsky, 1997), which in turn dictates the timing and alignment of the chromosomes to the spindle. Disruptions in the timing, assembly or the alignment of chromosomes it will be detected by the cells checkpoints system (Steuerwald et al., 2001). A signal is transduced, or initiated, which stops the assembly until the correct spindle structure can be formed. If this mechanism of checkpoints does not operate, incorrect alignments, non-bipolar spindles or timing inconsistencies may occur. This will lead to certain chromosomal aneuploidies. As oocytes age the surveillance systems begin to breakdown, incorrect spindle assembly or chromosome alignments result, which lead to aneuploidies in older oocytes (Steuerwald et al., 2001). However, FISH data show that this system is apparently not that precise in human oocytes, including those from younger women, given the very high frequency of chromosomal aneuploidies for all chromosomes shown in human oocytes, including those involving multiple chromosomes. Aneuploid oocytes have little developmental competence but are capable of fertilization and development, appearing morphologically normal. Aneuploidy most likely occurs during the maturation process of the oocyte.

It has been suggested that disruption of the meiotic spindle, which will result in abnormal development and thus reduced competence, can contribute to aneuploidy. Experiments on cooling immature and mature oocytes for cryopreservation have given insights into the dynamics of spindle structure and the consequences of its disruption. Since a large component of the spindle is tubulin, which depolymerizes as the temperature drops, cooling or chilling oocytes at any stage of development can have a profound effect on the spindle. This has been demonstrated in mouse (Pickering and Johnson, 1987),

bovine (Wu et al., 1999) and human (Sathanathan et al., 1988; Pickering et al., 1990; Almeida and Bolton, 1995; Wang, et al., 2001; Zenes et al., 2001).

During cooling the spindle begins to disrupt, seen initially as displacement of the chromosomes and then by the disruption and disappearance of the microtubules running from pole to pole, leading to shortening of the spindle. During cooling the chromosomes do not become dispersed in the cytoplasm but they can become disorganized or rearranged on the spindle. They remain anchored in one area, at the metaphase plate (Zenes et al., 2001), most probably through their kinetochores, which are anchored at the microtubule organizing centers. Lack of chromosome scattering has been found in other studies of cooling bovine (Wu et al., 1999) and human oocytes (Pickering et al., 1990; Van Blerkom and Davis, 1994; Almeida and Bolton, 1995; Bernard and Fuller, 1996). These experiments suggest microtubule disruption, without kinetochores loss, *in vivo* is not a cause for aneuploidy and that aneuploidy must have its origin in some other mechanism.

In fresh, non-cooled oocytes, at approximately 40 h post-hCG, it has been shown that between 17 and 22% (Battaglia et al., 1996; Park et al., 1997; Zenes et al., 2001) of oocytes of younger women, and up to 79% of aged oocytes or those from older women (Pickering et al., 1988; Battaglia et al., 1996) have abnormal spindles. The abnormalities range from disorganized to tri- and tetrapolar spindles with occasional instances of displaced chromatids, which may be related to a kinetochore or centromere dysfunction.

The spindles of oocytes can be visualized at different levels of resolution in fixed specimens by immunocytochemistry, or immunofluorescence (Gorbsky et al., 1990; Lee et al., 2000; Zenes et al., 2001) or in living oocytes by polarizing light microscopy (Oldenbourg et al., 1998; Katoh et al., 1999). Polarizing light microscopy allows the ordered structure (birefringence) of an object to be measured. Light travels at different speeds down or through samples. When highly ordered structures, such as spindle microtubules are bundled in arrays, light travels through the path of the array at a different speed than it would through non-bundled. This differential produces birefringence that can be resolved and visualized (by differential color) by image analysis of the polarized image. If the array is disrupted the polarized image will also be disrupted. Thus, intact ordered spindles can be visualized whereas spindles that are disassembling or disorganized cannot be detected in living oocytes. The use of polarized light microscopy could give an indication of the oocytes competence relative to the spindle, since spindle integrity is necessary for continued development.

The metaphase II spindle is normally located on the periphery of the oocyte, in the vicinity of the first polar body and near to the inner surface of the cell membrane (Sousa and Tesarik, 1994; Edwards and Beard, 1997; Silva et al., 1999; Hardarson et al., 2000). Evidence from mouse oocytes suggests

that the spindle moves away from the polar body in a time dependent manner (Kono et al., 1991). The use of fixation and polarizing microscopy has shown this is the case in hamster (Silva et al., 1999) and human oocytes (Hardarson et al., 2000; Wang, et al., 2001). In these studies the metaphase spindle and the first polar body were in the same hemisphere of the oocyte but the spindle was rarely located directly under the polar body. By using the center of the oocyte as the pivot, the angle between the first polar body and the metaphase spindle has been calculated to vary from 0° - 90° in oocytes at the time of ovulation (retrieval) (Hardarson et al., 2000; Scott, 2001b). Since the spindle is on the inner cell membrane and the oocyte is spherical, the angle between the polar body, central pivot, and the metaphase spindle could increase with time as the spindle moves away from the polar body (Kono et al., 1991). This could indicate the competence of the oocyte since at the time of ovulation the angle should be small, indicating little movement, and the further the spindle has moved the older or more aged the oocyte is.

It has been shown that there is a difference in the angle between the spindle and first polar body in fresh (in vivo matured) versus in vitro matured oocytes (Hardarson et al., 2000) (see Fig. 13.2). The mean angle was 42° degrees in in vivo matured oocytes at 40-42 h post-hCG, and 27° in in vitro matured oocytes, suggesting culture related delays in the progress of oocyte maturation. In vitro maturation is also correlated with decreased developmental competence. The position of the second polar body also indicates the position where the metaphase spindle was located. The further it is away from the first polar body the further spindle should have migrated prior to fertilization, or that the first polar body has migrated in the perivitelline space. It has been shown that fertilized oocytes showing small or large displacement of the 2 polar bodies have equal developmental competence (Garello et al., 1999; Silva et al., 1999). From these reports it is hard to correlate spindle position with competence, although spindle integrity is necessary for continued development.

OOCYTE COMPETENCE AND FERTILIZATION

At ovulation, the meiotic maturation of the oocyte is arrested at metaphase II, and is reinitiated with sperm penetration resulting in the completion of meiosis with the abstraction of the second polar body. This stage of resumed meiosis is accompanied by the induction of a series of calcium spikes and waves (Carroll et al., 1996; Carroll, 2001). Without this timed sequence of calcium waves, the fertilization process, which most notably includes pronuclear evolution, will not progress normally and development will be impaired. Studies with the mouse model have shown that an intact spindle is

essential to both initiate and sustain these calcium waves and is required for the completion of the final phase of meiosis (Winston et al., 1995).

While immature human oocytes, including those at the germinal vesicle stage, can be penetrated by a spermatozoon (Van Blerkom et al., 1994) only those that have completed meiotic maturation are actually fertilizable as demonstrated by the ability to promote pronuclear development. In lower-order animals and in some mammals, the site of sperm entry is directed or localized. In mouse fertilization, the sperm attach to micro villous sites on the oocyte surface, and in this species, the oolemma in the vicinity of the first polar body is devoid of microvilli and thus unable to bind sperm. As a consequence, the site of sperm penetration does not occur in proximity to the metaphase II spindle and binding and penetration only occur when the oocyte is competent to receive the sperm. In contrast, a similar microvilli-free area has not been detected in other mammals, including the human (Pickering et al., 1988; Santella et al., 1992). By constructing cytoplasts from human oocytes it was shown that sperm could bind and enter an oocyte at any point (Levron et al., 1995), including the region of cytoplasm occupied by the MII spindle. Coupled with the data that immature oocytes can be penetrated by sperm (Van Blerkom et al., 1994) the lack of a defined sperm-binding site cannot be used for oocyte competence estimates.

The distribution of mitochondria in the oocytes is also important for fertilization. *In vivo*, mitochondria respond to secondary signaling pathways. The result is proton pumping across the mitochondrial membrane that results in both a pH gradient and a membrane potential. Using a staining technique, which visualizes mitochondria that are polarized, or capable of actively pumping protons, has indicated that the distribution of polarized mitochondria in oocytes and embryos is not uniform but has an ordered pattern (Van Blerkom et al., 2002). It was shown that in immature (GV) and early MI oocytes the mitochondria that have low polarization (membrane potentials) are located in a ring around the nucleus. Mitochondria remain in a dense ring around the nuclei but a band of mitochondria appear in the pericortical area as the oocyte progresses to the MII phase. Although there are high polarized mitochondria in the polar body there are none directly under it. A band of polarized mitochondria spreads out from the site of the polar body but does not spread all the way to the opposite pole, which is devoid of polarized mitochondria. There are no polarized mitochondria wherever there is cell contact, either in the oocyte with contact between granulosa cells or polar bodies or in cleaving embryos where there is contact between blastomeres (Van Blerkom et al., 2002). Clearly the distribution and activity of mitochondria are differential and play a large role in the developmental potential of an oocyte or embryo. When this pattern of distribution was disrupted no or abnormal development ensued.

Calcium binding proteins actively sequester calcium into the intraorganellar space, such as the endoplasmic reticulum (ER), which also contains calcium-buffering proteins. The ER and the cytosol space are the two largest stores of calcium and in the oocyte there are also sub-oolemmal granules, which store calcium. Calcium-binding proteins release calcium across the cell membrane into the cell cytosol for signal transduction. Oocytes and embryos release calcium from stores through a non-excitatory or slow pathway, which is driven by inositol 1,4,5-triphosphate (InsP₃) and its receptor, InsP₃R, and another receptor, the ryanodine receptor (RyR). In all cells there are two mechanisms for releasing calcium, the G protein-coupled receptors and the tyrosine kinases (RTK's), both of which release InsP₃. The G proteins activate phospholipases and the RTKs stimulate the conversion of phosphatidylinositol 4,5 bisphosphate (PIP₂) to InsP₃ and diacylglycerol. The InsP₃ acts as the second messenger to bind the tetrameric receptor, InsP₃R, which spans the ER and releases calcium from ER-associated calcium-binding proteins. InsP₃R is a homotetramer and each subunit binds one InsP₃ molecule through a positively charged Arg/Lys rich, N terminal region. At low calcium levels the receptor is insensitive to InsP₃ but is most sensitive between 0.5-1.0 uM, the physiologic levels of cells (Lechleiter et al., 1991; Lechleiter and Clapham, 1992; Berridge, 1993; Clapham, 1995; Stehno-Bittel et al., 1995).

Calcium wave propagation is both time and spatially regulated. In InsP₃ calcium release the InsP₃ is generated by G protein-linked receptor stimulation of phospholipase and diffuses rapidly to the cell and binds the InsP₃R for a few minutes before being degraded. InsP₃R's release calcium in certain spots, "hot spots," where the local calcium concentration is high, there is a high concentration of InsP₃R or a high concentration of InsP₃. The calcium being released from the ER lags behind the InsP₃ and diffuses to adjacent sites where it causes a shift in the dependence of InsP₃R curves to its peak and induces calcium release thus setting up the wave front. Local calcium release generates high concentrations of calcium at the mouth of the channels and inhibits the channel (feed back mechanism). Calcium ATPase pumps remove calcium from the cytoplasm. Since the diffusion of calcium is 3 dimensional this series of release and sequestration results in waves over a large 3 dimensional area in complex patterns (Lechleiter et al., 1991; Atri et al., 1993). The most complex patterns of waves and spirals are seen in large cells such as oocytes (Van Blerkom et al., 2002).

At sperm penetration the sperm induces an increase in intracellular free calcium levels. This event triggers the transition from oocyte to embryo and is therefore fundamental to development (Carroll et al., 1996; Carroll, 2001). The fertilizing spermatozoa releases "factors" which trigger calcium release resulting in a timed series of calcium oscillations and the exocytosis of

cortical granules (CG), which prevent polyspermy (Abbott et al., 1999). After the initial release, subsequent low amplitude/frequency calcium oscillations that persist for hours. The calcium waves originate from the site of sperm entry and follow the pattern of InsP₃ release and binding.

Only MII oocytes can initiate the calcium release. Immature oocytes are unable to undergo cortical granule (CG) release, which is dependent on InsP₃ mediated elevation of calcium (Ducibella and Buetow, 1994; Abbott et al., 1999). This system functions as a regulator of fertilization. The sperm head contains sperm-born oocyte-activating factor (SOAF), which is a serine protease. The involvement of SOAF in the induction of calcium transients is through proteolytic processing of SOAF's from the sperm head (Perry et al., 2000). The sperm is not totally responsible for these events since the mature MII oocyte contains a factor, which mediates the events stimulated by the sperm (Tang et al., 2000). The calcium waves initiated by the entry of the sperm are seen in all animals models studied to date and are probably highly conserved. The first increase in calcium at activation is in two steps, with maximum calcium concentrations at the mid point of the waves. This may indicate that two mechanisms of calcium release are active, namely the InsP₃ and RYR systems. The first wave is initiated at the sperm entry site and moves to the opposite side/pole of the oocyte. The second wave follows the same path. Movement or rotation of the cytoplasm follows this wave (Tang et al., 2000). The movement follows the general direction of the wave. This particular series of calcium oscillations, initiated at sperm entry, continue through the second meiotic division and end at the stage of pronuclear formation, with the amplitude of the waves becoming attenuated with time. The speed of the waves also increases as fertilization progresses. With each wave there is further cytoplasmic displacement in the oocyte. The point of wave initiation moves around the oocyte away from the point of sperm entry to the opposite pole (Tang et al., 2000). The correct and sequential release of calcium is essential to development and the competence of the newly formed embryo but it is not possible to quantify it. If fertilized oocytes are not progressing the ability of either the sperm or the oocyte to initiate a full series of calcium waves may be the source of reduced competence.

PRONUCLEAR STAGE EMBRYOS

Once the sperm enters the oocyte a rapid and series of morphodynamic events occurs leading to the second polar body extrusion and the formation of the pronuclei. As with first polar body, the second polar body represents a unique form of unequal cell division and occurs close to the metaphase plate to limit the amount of cytoplasmic loss. The sperm head begins to decondense prior to the extrusion of the second polar body. The sperm head

moves from the outside of the oocyte (cortex) into the interior fairly rapidly (Van Blerkom, et al., 1995). The male pronucleus appears near the center of the oocyte with the female pronucleus near the site of the metaphase spindle (Payne et al., 1997). The position of the sperm nucleus determines where the male and female become juxtaposed, with this being in the periphery of the oocyte if the sperm has entered the oocyte near the spindle and in the center if it has entered in the hemisphere not containing the spindle (Van Blerkom, et al., 1995). The second polar body does not degrade or fragment in the same manner as the first polar body but the DNA within it does degrade. Both *c-mos* and MAPK are inactivated at fertilization (Verlhac et al., 1994) so this pathway for degradation is not active in the second polar body. The second polar body, which is haploid, has also been used in nuclear transfer experiments in mice with live offspring being reported (Wakayama et al., 1997). It is linked with axis formation in the embryo and its persistence through to the blastocyst stage has been demonstrated in the mouse, where it defines the embryonic-abembryonic axis (Gardner, 1997).

As the sperm head de-condenses an aster of microtubules forms from the sperm centrosome, promoting microtubule growth. As the aster forms there is a "flare" within the cytoplasm, seen by time lapse video cinematography, and presenting as differential cytoplasmic density over time. This could be associated with aster growth (Payne et al., 1997) and the sperm nucleus moving towards the center of the oocyte. In most species the male and female nuclei are brought together in the center of the oocyte through the movements of microtubules (Schatten, 1994) and the pronuclei become visible. In the human, the sperm head is in the proximity of the MII spindle shortly after entry (Van Blerkom, et al., 1995) and the formation of the male and female pronuclei occur close together. If they are not close, the microtubule array exists to align them, but is more a back up system to ensure the correct alignment (Van Blerkom, et al., 1995).

After completion of DNA replication and condensation into chromosomes, the opposed pronuclear membranes breakdown and the maternal and paternal chromosomes mix and become aligned on the first mitotic spindle. The first cleavage division ensues, resulting in a 2-cell embryo, which is the completion of the fertilization process. .

In the mouse, the site of sperm entry has been suggested to have an influence on the axis of the first cleavage division (Gardner, 2001; Piotrowska and Zernicka-Goetz, 2001; Plusa et al., 2002) although it has also been shown to have no correlation (Davies and Gardner, 2002). Parthenogenetically activated mouse oocytes, with no sperm entry site, can also begin development, although they suffer many abnormalities after implantation. Some of these relate to axis formation and are typified by folding anomalies such as cleft palate, failure of the neural tube to close, duplication of head

structures, which may point to the need for early axis definition by sperm entry but more likely the incorrect initiation of calcium waves.

In the mouse the first cleavage plane is typically down an axis defined by the position of the second polar body (Gardner, 2001) such that the polar body ends up in the furrow of this plane. This first axis formation has not been well defined in the human as yet.

Apart from the “flare” associated with fertilization which is seen on time lapse video screening, there is evidence of cytoplasmic streaming or movement as seen by differential cytoplasm distribution in the pronuclear embryo (Payne et al., 1997; Scott and Smith, 1998). This is reminiscent of the streaming seen in most lower order animals, which is essential for development (Seydoux and Fire, 1994; Kloc and Etkin, 1995; Edwards and Beard, 1997; Scott, 2001b;) and where it is associated with ribonucleic protein redistribution (Seydoux and Fire, 1994; Kloc and Etkin, 1995). A type of streaming has been documented in human oocytes and pronuclear embryos and has been characterized as a “halo” effect (Payne et al., 1997; Scott and Smith, 1998). Both hamster (Barnett et al., 1996) and mouse (Muggleton-Harris and Brown, 1988) oocytes show this appearance of streaming which has been attributed to differential localization of mitochondria within the cytoplasm (Barnett et al., 1996; Muggleton-Harris and Brown, 1988) (Figure 13.3). This movement can be seen in human pronuclear embryos on time-lapse video recordings, although it is not necessarily directional (Hardarson, unpublished data).

The male pronucleus also rotates on the central axis (Van Blerkom, et al., 1995). When chromatin in the two pronuclei decondenses, the female nucleus has polarized condensation in its distal membrane, i.e., the side of the nucleus furthest from the oolemma. The chromatin in the male nucleus decondenses on the outside of the pronucleus, at the site of the sperm tail attachment, the region that also encompasses the centrosome. As the pronuclei become opposed, the male nucleus can apparently rotate such that the chromatin of the two pronuclei is aligned at the region of the membrane juxtaposition, which places the centrosome between the nuclei (Van Blerkom, et al., 1995). The streaming or movement seen in the pronuclear embryo may also be indicative of this rotation occurring. The importance of chromatin alignment with respect to competence may be related to developmental arrest associated with abnormal alignment, and could result in chromosomal mosaicism or multinucleation at the 2-cell stage if the condensed chromosomes are not appropriately juxtaposed with respect to the position of the first mitotic spindle.

Normal development is associated with the bulk of mitochondria accumulated around the opposed pronuclei with a corresponding clearing in the pericortical cytoplasm (Van Blerkom et al., 2000). This is can also

contribute to the halo or clearing used in ‘zygote scoring’ systems (Payne et al., 1997; Scott and Smith, 1998). Whether the observed halo is cytoplasmic streaming allowing movement or the redistribution of mitochondria or both, has not been established equivocally. It is probably both, but the subtlety of the 2 cannot be distinguished at a light microscope level.

For clinical applications oocytes and embryos cannot be stained to identify active mitochondria and distribution patterns. The halo effect can be seen with differential and phase contrast optics and can be used as an indicator that the events of cytoplasmic streaming and nuclei alignment may be occurring correctly.

THE NUCLEOLUS AND NUCLEOLAR PRECURSOR BODIES

The nucleus governs much of what happens in a cell, and within this structure are the nucleoli, a major site of RNA and ribonucleoprotein biosynthesis. Nucleoli are the sites where ribosomal genes (rDNA) are transcribed and become associated with ribosomal proteins. Nucleoli are found in all actively dividing eukaryotic cells. They present as dense ovoid structures in which appear and disappear at specific stages of the cell cycle. There are generally between 2-to-5 per human nucleoli with equality between daughter cells. Nucleoli develop at the “nucleolus organizing regions” (NORs) of chromosomes where tandem repeat sequences coding for ribosomal RNA (rDNA) are located. These are also the sites where RNA and pre-ribosomal synthesis take place and develop into nucleoli. Structurally, nucleoli consist of 3 components, a dense fibrillar element (DFC), a fibrillar center (FC), and a granular component matrix (GC). rDNA transcription is associated with the DFC, which is attached to the DNA, but does not require the FC, which are located near the DFC. rDNA transcription is restricted to foci on the DNA and FC’s act as structural centers or sites for transcription and storage of inactive transcription factors that when activated, initiate rDNA expression. The GC is composed of pre-ribosomes (Schwarzacher and Mosgoeller, 2000).

In mitotic cell cycles there are more nucleoli present at the beginning of the cell cycle (G1 phase), but fusion occurs as the cell cycle progresses so that at the S phase, only 1 or 2 relatively large nucleoli occur (Goessens, 1984). Daughter cells display synchrony in both the number and form of fusion of the nucleoli with asynchronous fusion resulting from aberrant chromosomal function (Goessens, 1984). Asynchrony between daughter cells with respect to the number of nucleoli was first described as a means of differentiating cervical cancer cells from normal ones. Other forms of cancer cells display more than the expected numbers of NOR’s, or NOR’s of differing, abnormal or unequal sizes in daughter cells. The nucleolus has also been implicated in

the process of aging where they progressively present as fragmented, dense elements (Guarente, 1997). The nucleolus is also the site of many of the mitogenic and growth regulatory proteins thought to be involved with cell cycle control (Pedersen, 1998).

Oocytes in antral follicles actively synthesize RNA and have well defined nucleoli. This is essential in order for the oocyte to attain full meiotic competence through oocyte growth (Motlik, et al., 1984). As the oocyte matures RNA synthesis decreases and the nucleoli become small and scattered (Crozet et al., 1986). At the germinal vesicle stage there is one large nucleolus that is clearly visible in the center of the GV. At metaphase II and the fertilized oocyte stage the nucleoli are present as precursor bodies, NPB (Tesarik and Kopecny, 1990), seen as dense structures in the nucleus. NPB are not full nucleoli, consisting of only the fibrillar centers (FC's), and these structures do not synthesize rRNA. In early embryos, the formation of nucleoli occurs over several mitotic cell cycles with the preformed NPB's gradually transforming into functional nucleoli through interactions with the rDNA genes (Laurincik et al., 2000). This needs to be sequential and regulated. Full nucleolus structure and function returns as the embryo cleaves (Tesarik and Kopecny, 1989, 1990) and becomes fully active, usually when developmental regulation switches from oocyte-derived to embryonic-derived with the activation of the embryonic genome (Goessens, 1984; Flechon and Kopecny, 1998; Hyttel et al., 2000).

An experimental model, which illustrates the consequences of disruption of parts of the nucleolar-cycle, is what is observed in the construction of embryos in which nuclei were transferred from somatic cells. After nuclear transfer, extensive re-modeling of the nucleolus occurs, even to the point where they deconstruct and return to the type of NPB's characteristic of the early embryo (Kanka et al., 1999). The subsequent re-formation is essential for correct rRNA production and embryo development. In many instances the remodeling is incomplete or aberrant, leading to abnormal development, underscoring the importance of the sequential and functional construction of nucleoli during development. Another study underlying the importance of correct nucleolus formation is illustrated when mouse or human GV oocytes are cooled. On cooling the nucleolus (generally only 1 or 2) began to disaggregate into NPB's. After freezing, thawing and re-hydration the NPB's reformed into nucleoli, which then disappeared as the germinal vesicle broke down with oocyte maturation. Without reformation of the nucleolus, GV breakdown could not occur.

It has also been shown that the chromatin within human pronuclei is polarized, i.e., equatorially localized at the site of pronuclear membrane opposition (Van Blerkom, et al., 1995). It was suggested that malalignment could result in chromosomal aneuploidies and mosaicism during cleavage if

pronuclear embryos with this phenotype underwent syngamy (Van Blerkom, et al., 1995). Since the NOR's and NPB are associated with strands of chromatin, non-alignment of chromatin could also lead to non-alignment of the NPB.

The pronuclear stage is the first developmental point at which a genetically discreet embryo exists. In clinical IVF, the pronuclear embryo is also the first stage of embryogenesis routinely examined in the laboratory to confirm fertilization, and as such, affords an ideal time for competence selection based on the parameters that may constitute or be associated with viability. These would include the presence of two pronuclei and two distinct polar bodies, indicating monospermic fertilization and completion of meiosis. Further, the NPB's, which are readily detectable by light microscopy, have the potential to add additional specificity to competence scoring as their progression to 2-to-5 per pronucleus and their equatorial alignment at the region of pronuclear juxtaposition /nuclear junction may indicate normality of both the female and male nuclear components and the correct rotation and normal alignment of the male pronucleus with respect to the cleavage plane of the first mitotic division.

ESTABLISHMENT OF COMPETENCE DURING CLEAVAGE

It appears that in mammalian embryos examined to date, the first cleavage division is meridional, and always unequal (one cell is always slightly bigger than the other) and non-random, resulting in cells that are not spherical but rather elliptical. If the pronuclei are not correctly aligned on the cleavage plane they are capable of rotation in order for division to occur (Scott, 2001b). The cleavage plane is defined by the position of the second polar body and the pronuclei and after the first cleavage division, the two-cell embryo can be considered to have a "top" delineated by the second polar body and two 'sides'. The long axis coincides with the polar axis of the embryo. The two-cell embryo is unable to rotate freely inside the zona pellucida. In the mouse, the second polar body is anchored to the embryo via a cytoplasmic tether which is never severed, remains attached to only one of the 2 blastomeres and throughout subsequent preimplantation stages, persists on the outside of the embryo (Gardner, 1997; Piotrowska and Zernicka-Goetz, 2001) to become located at the apex of polar trophectoderm, i.e., the area of the blastocyst where the inner cell mass is located (Gardner, 1997).

The second cleavage plane in the two-cell embryo appears to involve one meridional and one equatorial division. The timing of cell division is such that one blastomer divides slightly ahead of the other with the second rotating onto the plane forming an embryo with a tetrahedron shape (Edwards et al., 1984; Gardner, 2001). Deviations from this pattern may indicate that the putative

orientation-associated signals at the 1 and 2-cell stages could have been out of sequence. This may also lead to 4-cell embryos in which blastomeres are arranged in a linear rather than tetrahedral geometry. However, there is no definitive evidence to suggest that, deviations from the normal tetrahedral orientation are developmentally significant. In normal human development, temporal asynchrony in blastomere divisions is observed at least up to the 8-cell stage (Roux et al., 1995). In the 2- to- 8-cell embryo only three sizes of blastomeres can exist and which correspond to the cell stages. However, at any time point there may be more than one cell size. For example at the 3 cell stage or 5/6 cell stage there will be 2 cell sizes in the embryo, which is a normal occurrence and allows the spatial orientation of the cells within the zona.

Owing to the geometric organization of the late cleavage/early morula stage embryo, two distinct cell types can be identified based on location: inside cells and outside cells (Johnson and Ziomek, 1981). At cavitation and early blastocyst formation the outside cells are totally committed to form trophectoderm. The trophectoderm cells proliferate in the early blastocyst but not in the expanded blastocyst, where the cells in the mural area (opposite the ICM) transform into giant cells (Gardner, 2000), indicated by the presence of small evenly spaced cells on the periphery of the embryo. As the blastocyst expands these peripheral cells elongate and become the so-called giant cells. The cells in the polar trophectoderm (which overlies the ICM) remain mitotically active and there is a flow of cells from this region to the mural trophectoderm in a directional or polarized manner (Gardner and Nichols, 1991). At the junction between the polar and mural trophectoderm, the migrating cells elaborate cytoplasmic processes that extend into the central portion of the ICM (Flemming et al., 1984). These extensions are transient elements which are withdrawn as trophectodermal cells migrate to the mural region. If they are not withdrawn it may indicate incompetence of the trophectoderm, which could influence implantation. In the mouse, the second polar body is always associated with the polar trophectoderm (Gardner, 1997, 2000), and this orientation defines the embryonic-abembryonic axis, which is the first defining axis of gastrulation in the fetus (Smith, 1980; Smith, 1985), i.e., the true dorso-ventral axis and the true left-right axis for the embryo/fetus. If there have been disruptions to the axis formation in the first cleavage, it may be manifested in the incorrect positioning of the second polar body in the blastocyst which may also lead to developmental incompetence at implantation and later fetal development.

CLINICAL APPLICATIONS OF OOCYTE AND EMBRYO DEVELOPMENTAL BIOLOGY

OOCYTE MORPHOLOGY

There is insufficient data on the heterogeneity of human oocytes subjected to high dose of gonadotropins. The clinical IVF experience demonstrates that within cohorts, MII oocytes obtained after ovarian hyperstimulation are developmentally heterogeneous. MII oocytes can also display morphological heterogeneity and with a few exceptions, it is difficult to use morphology as a definitive indicator of competence. For the human, it is unclear what morphology exhibited by newly retrieved oocytes is in fact normal and therefore useful as a standard phenotype.

There are many factors that can be taken into account such as a granular appearance to the ooplasm, the presence of vacuoles and pit like structures discerned with differential contrast microscopy, or small inclusions in the perivitelline space. Some reports link these phenomena with decreased implantation potential (Serhal et al., 1997) while do not (De Sutter et al., 1996). However, keeping track of these anomalies may give an indication of what has happened during oocyte development in the follicle and predict outcome (Van Blerkom, 1990; 1994; Van Blerkom and Henry, 1992; Meriano et al., 2001).

The laboratory environment the oocyte is subject to could have a profound effect on its later developmental capacity, although to date no definitive studies have been done to discern these. The medium in which oocytes are fertilized may play a role in the activation of certain pathways or genes in the oocyte, effecting downstream developmental events, however, to date a wide variety have been used, all leading to similar results (Rawlins, 2001; Scott, 2001a). Granulosa cells actively metabolize glucose (Donahue and Stern, 1968), forming pyruvate, which is required by the fertilized oocyte (Biggers et al., 1967). Many media formulations are devoid of glucose, since it has been assumed that glucose is inhibitory to early development. However, the negative effects of glucose are mediated by inorganic phosphate (Scott, 1999). Inorganic phosphate is required for the transport of glucose into the oocyte by facilitative transport, it inhibits the allosteric regulation of hexokinase by its product, glucose-6-phosphate, which in turn removes a natural block to glucose flooding into the cell, and it inhibits pyruvate kinase, which will prevent pyruvate being cycled into the OXPHOS cycle, thus depleting the oocyte of ATP (Scott and Whittingham, 1996; Scott, 1999, 2002). To date the removal of glucose from fertilization medium has not had any negative effect on fertilization rates or embryo development (see Biggers, this volume). However, since both granulosa cells and later developmental stages of the

embryo require glucose, it may be more beneficial to utilize a media with some glucose but devoid of inorganic phosphate.

POLAR BODY MORPHOLOGY AND COMPETENCE

Results from some studies suggest that the morphology of the polar body is related to fertilization and implantation competence in clinical ICSI (Xia, 1997; Ebner et al., 1999, 2000). In these studies, it was reported that a fragmenting (polar body that appears to be 2 intact small polar bodies) and degenerating polar body (clearly degenerate, dark, small pieces) at the time of ICSI resulted in decreased fertilization, development and implantation rates. Degradation of the first polar body is a normal phenomenon related to the age of the oocyte. An early event of disintegration could indicate decreased developmental competence, as has been reported. If the polar body is already fragmenting and degrading at the time of ICSI, at 40 hours post hCG, it could indicate a disruption of an event earlier in the process of oocyte maturation. During routine IVF cases this could be achieved by spreading the oocytes (Veeck, 1988). This has its limitations since the granulosa cells could mask the true identification of fragmentation.

SPINDLES: POSITION AND INTEGRITY

Using the Polscope technique it was shown that oocytes not showing a birefringent spindle prior to ICSI had reduced fertilization rates and were slower to develop in vitro than those with detectable spindles (Wang, et al, 2001). In an earlier study using immunofluorescence and natural cycle oocytes, Battaglia et al. (Battaglia et al., 1996), showed that this phenomenon was age related. The spindle abnormalities attributed to aging and detected with immunofluorescence were abnormal shapes, disorganized microtubule placement and finally disorganized placement of chromosomes on the metaphase plate (Battaglia et al., 1996). In vitro aging has also been shown to affect the spindle, causing a lack of birefringence (Wang, et al, 2001), which should be taken into account when using this technique to assess oocytes.

Abnormalities in spindles could lead to impaired development, which may be recognized as reduced rates of fertilization and development, even without the use of spindle visualization techniques such as polarized light microscopy. Those with very disrupted spindles may not even complete the fertilization process correctly (Winston et al., 1995) and either self-select or can be selected for very early after fertilization. As age is already documented as the major factor in reduced reproductive success and increased rates of aneuploidy, it is assumed that increased spindle abnormalities will also exist (Steuerwald et al., 2001). As many embryos with no visualized spindle did

progress normally through the implantation stages to the blastocyst (Wang, et al, 2001) it is necessary to know if such embryos have normal implantation potential. If competence is retained at the cleavage or blastocysts stage in embryos derived from oocytes where no birefringent spindle could be detected by PolScope imaging, the validity of this approach to oocyte selection would be suspect in a clinical setting. However, if spindle disruption through cooling, even minimal, can induce permanent or transient disruptions in spindle organization Polscope imaging may have value in assessing the extent to which a human oocyte can be cooled without damage, identify spatial and temporal aspects of recovery, and in oocyte age) may afford, if any, the best protection against disruption or recovery. The visualization of spindles could also be used as a means of monitoring laboratory-handling techniques, to ensure that spindle disruption is not iatrogenic.

The use of the Polscope to monitor spindle position for ICSI may not be practical or relevant in a clinical setting. Since the spindle is generally in the same hemisphere as the polar body and it moves away from the oolemma in a time dependent manner, knowing the age of the oocyte relative to hCG should indicate the general area in which the spindle is located. This can be avoided by performing ICSI with the polar body placed in an approximate 6 or 12 o'clock position. If the spindle has moved so far as to be at a 90-degree angle, the oocyte will most likely be aged and not develop in any case. Further, if the spindle has moved so far from the location of the first polar body, it most likely will have begun to disintegrate, another indication of time since completion of metaphase I (Figure 13.2).

The use of noninvasive techniques to assess oocyte spindles may have a place in human IVF, not for ICSI, but for selecting oocytes with the most potential in older patients. Where it is already known from invasive studies that a high percentage of MI and MII oocytes have abnormal spindles. However, the labile nature of microtubules makes this a delicate procedure open to the dangers of many artifacts and inconclusive data. If the temperature can be controlled, not only for assessing the spindle but also for all procedures in oocyte handling, a true correlation with spindle abnormalities, embryo viability and implantation could be elucidated. Further, exact timing of oocyte maturation should be known in order to make any correlations with spindle shape. If studies can demonstrate conclusively, with large numbers of oocytes, that oocytes with disrupted/no spindles can fertilize, form good-grade cleaving embryos and blastocysts such that they cannot be eliminated on the basis of development and morphology, but that they have absolutely no ability to implant, regardless of the stage of embryo transfer, spindle assessment will be a powerful tool. Further, the morphology of the human metaphase II spindle needs to be described in detail with the range of abnormalities resulting in viability and non-viability described and quantified. Thus while

Polscope imaging may have a place in IVF, its application must be considered experimental at present.

SPERM ENTRY SITE

The further away from the metaphase spindle that the sperm enters or is placed, the further the microtubules will need to grow in order to pull the female pronucleus into a central position. If sperm entry is directed as in lower order animals, this will ensure optimal movement. It has been reported that in ICSI that the placement of sperm in a central area just outside of the hemisphere containing the polar body gives optimum results (Garello et al., 1999; Van der Westerlaken et al., 1999; Blake et al., 2000). However, considering the movement of the spindle, the fact that the oocyte is spherical and in most instances exact timing of completion of MI is not precisely known, the use of 6, 7, 11, or 12 o'clock positions should not effect the results. In one study the position (6 or 12 o'clock placement) had no effect on fertilization and outcome was shown to be largely operator dependent (Van der Westerlaken et al., 1999). With the first polar body in an approximate 6 or 12 o'clock configuration the sperm would be positioned closer to the site of the spindle, a notion that has been documented by Hardarson et al. (Hardarson et al., 2000).

Thus for ICSI cases, the optimal time of cumulus and coronal cell denudation is at about 40 h post-hCG. Any breaking or clearing in the granulosa cells should be noted in relation to the polar body as this may be a directed or preferential site of directed sperm entry. The polar body should be assessed for integrity. Fragmentation and degradation may indicate a possible premature aging of the oocyte, or an early/abnormal completion of meiosis I. Since non-disjunction largely occurs at MI, fragmenting polar bodies may indicate aneuploid oocytes.

NUCLEI AND NUCLEOLAR PRECURSOR BODIES

Fertilized oocyte or pronuclear scoring that includes nuclear alignment and NPB morphology has been successfully used to increase rates of implantation and to reduce the number of embryos required for transfer (Ludwig et al., 2000; Scott et al., 2000; Wittemer et al., 2000; Balaban et al., 2001). Although a number of competence scoring models and schemes have been described (Scott and Smith, 1998; Tesarik and Greco, 1999; Scott et al., 2000), all rely on the same principles, which include pronuclear position and the number, size and distribution of NPB's. In general, normal fertilized oocytes are

considered to be those in which the nuclei are equal in size and centrally positioned with equal numbers of NPB's in each pronucleus. The NPB's are either aligned at the region of nuclear opposition, the nuclear junction or scattered evenly through the nucleus.

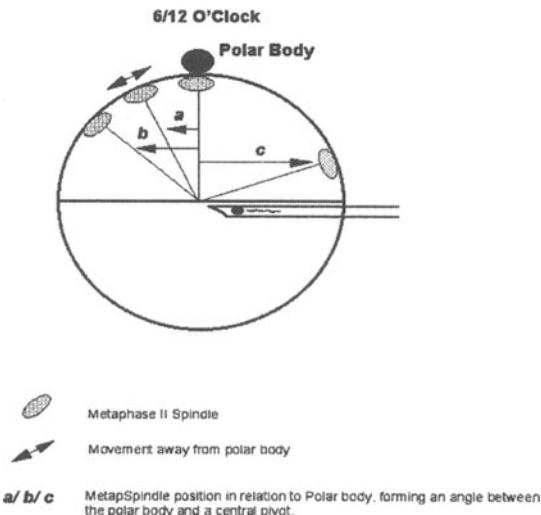


FIGURE 13.2. Spindle position during ICSI. At 38-42 h post hCG the spindle will be near to the polar body or at least in the same hemisphere. It will move away from the polar body with time. If the polar body is placed at 6 or 12 o'clock, relative to the holding pipette, the ICSI needle will not damage the spindle.

Abnormal fertilized oocytes, 16-18 h after fertilization, have nuclei that are not centrally located, or not juxtaposed, or exhibit grossly different sizes. Embryos resulting from these oocytes either arrest in development (Scott et al., 2000; Scott, 2002a) or have high rates of aneuploidy (Sadowy et al., 1998). Further, fertilized oocytes with 2 nuclei are of grossly different sizes have been shown to be aneuploid in the majority of cases (Munne and Cohen, 1998). Fertilized oocytes with fragmented nuclei also have poor developmental potential and are chromosomally abnormal. In most studies, only 10-12% of all fertilized oocytes have this nuclear morphology. Abnormal fertilized oocytes also present with NPB's that are of many different sizes, with inequality between the nuclei in terms of size, number and distribution of

NPB's (see Fig. 13. 3 for a schematic depiction of different fertilized oocyte scores).

Ideal fertilized oocytes should have equality between the nuclei with between 2-5 equal sized NPB per nucleus (Figure 13.4A, B). If they are lined up at the nuclei junction it is assumed that chromatin condensation is complete, the male pronucleus has rotated onto the axis and the chromosomes are all on the mitotic spindle. If the NPB's are scattered in the nucleus, it is assumed that the nuclei are synchronized but are not as advanced as those in which the NPB are aligned. Both of these types of fertilized oocytes have the highest developmental potential and implantation rates (Tesarik and Greco, 1999; Scott et al., 2000; Scott, 2002b).

If there is any form of inequality between the pronuclei the fertilized oocytes are considered abnormal (Figures 13.4C,D). The different forms of abnormal fertilized oocytes probably have different causes, however there is no definitive data on this. Fertilized oocytes with 1-3 large NPB on one side (generally they are on the nuclei junction) with more NPB that are scattered on the other side are delayed in the development of the male pronucleus with respect to condensation of the chromatin and rotation onto the axis (Tesarik et al., 2002). Fertilized oocytes with NPB's lined up on the nuclei junction in one nucleus with them scattered in the other but equal in numbers probably also have equality between the 2 nuclei but the male nucleus is again delayed in rotation onto the cleavage axis.

Fertilized oocytes that have very unequal numbers and sizes of NPB (large and small) have abnormalities that are unknown and may be of both male and female origin. A study of the different types of abnormal fertilized oocytes and how they cleave and grow to the blastocyst stage could elucidate the origin of the abnormalities. Fragmenting NPB and NPB or many different sizes in the nuclei may point to an aging effect in the oocyte (Guarente, 1997).

It has been shown that fertilized oocytes with unequal numbers, sizes or alignments of NPB have reduced development (Scott, 2002a), reduced blastocysts formation (Balaban et al., 2001; Scott et al., 2000; Scott, 2002a.) and reduced or no implantation potential (Tesarik and Greco, 1999; Ludwig et al., 2000; Scott et al., 2000; Wittemer et al., 2000; Balaban et al., 2001). Further, in a selected group of patients, embryos resulting from fertilized oocytes with abnormal NPB morphology had a higher incidence of chromosomal abnormalities, even in those that reached the blastocyst stage (Kahraman et al., 2002).

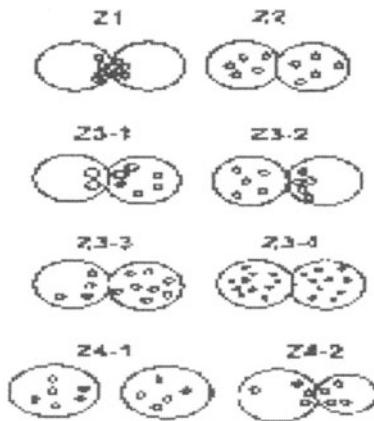


FIGURE 13.3. Schematic depiction of the NPB patterns in fertilized oocytes. Equality in number, size and distribution of the NPB's (Z1 and Z2) is the desired pattern. All other patterns (Z3), in which there is inequality of numbers, sizes or in distribution patterns are abnormal. Fertilized oocytes in which the nuclei are small, of very different sizes or are not close together are abnormal (Z4).

CYTOPLASMIC HALO

At the time of fertilization check 16-18 h post insemination/ICSI, differential cytoplasmic distribution in the fertilized oocyte cytoplasm can be seen. This has been termed the “halo” effect (Payne et al., 1997; Scott and Smith, 1998). This halo is most likely the cytoplasm movement related to mitochondrial and other cellular organelle redistribution (Barnett et al., 1996; Muggleton-Harris and Brown, 1988; Van Blerkom et al., 2000; Van Blerkom et al., 2002) and the rotation of the nuclei onto the polar axis (Van Blerkom et al., 2000; Scott, 2001b). Although movement of the cytoplasm cannot be seen in a single static observation, the occurrence of a dense central core with a clearing on the periphery of the embryo, can be used as an indicator that stage specific mitochondrial redistribution and cytoplasmic movement are occurring. The halo or this evidence of cytoplasmic movement has been correlated with increased developmental potential and implantation in human embryos (Payne et al., 1997; Scott and Smith, 1998). It has been shown that about 20% of fertilized oocytes do not have a halo and these have reduced in vitro developmental ability and blastocysts formation. Further, a higher proportion than the average population, had abnormal NPB profiles (Scott, 2002a). If the

halo is coupled with the fertilized oocyte score further embryo selection is possible.

CLEAVAGE

The final phase of fertilization is the first mitotic division and the formation of the 2-cell embryo. Early completion of this division has been associated with increased embryo viability (Shoukir et al., 1997; Sakkas et al., 2002), and can be detected as early as 22-24 hours after insemination. The 2-cell embryo should contain 2 elliptical cells with the second polar body in the cleavage furrow between them. If the polar body is outside of the plane defined by the furrow, it could indicate that the embryo derived from an aged oocyte, that there was a disruption of the meiotic spindle or that the patterning parameters have broken down. After 22-24 hours, a definitive time of the first cleavage division is difficult to establish and scoring of 2 or 4-cell embryo the following day may be meaningless with respect to the assessment of competence relative to the first dividing embryos. However, if observation times are set in a laboratory and all embryos are scored routinely at a standard time, a system for day 2 scoring could be established if the data is always correlated with outcome. One cell in the 2-cell embryo always divides ahead of the other, resulting in a 3-cell embryo with 2 different sized blastomeres (Roux et al., 1995). Rotation then occurs to form a tetrahedron of 4 cells within the zona pellucida, a spatial orientation essential for further development (Edwards and Beard, 1997; Gardner, 2001). Gross morphological assessments of day 3 embryos should include determinations of the uniformity and pattern of cleavage. There is a general consensus in clinical IVF that a high competence human embryo should have blastomeres of only one or two sizes (Roux et al., 1995), little or no fragmentation (see Van Blerkom, this volume), and most critically, no evidence of multinucleation (see Gerris, this volume). Intact embryos containing eight similar sized blastomeres have been consistently shown to have comparatively high implantation rates. Further, these embryos have a greater likelihood of developing to the blastocyst stage whereas those with fewer or more than 8-cells have a reduced developmental potential (Racowsky et al., 2000; Shapiro et al., 2000). Since embryos are on time clocks, very fast division means that the cleavage patterns are abnormal, the cell cycles are not functioning correctly, or that in fact the cells are really a-nucleated fragments. These embryos have poor prognosis after day 3, both in blastocyst development and in implantation (Racowsky et al., 2000; Shapiro et al., 2000).

However, it has also been shown that the day 3 morphology may not be a good predictor of blastocyst development in vitro (Graham et al., 2000) and that greater developmentally relevant differences can occur between embryos at the blastocyst stage than on day 3 (Milki et al., 2000). After approximately the 12-16-cell stage, compaction begins in the human embryo. If compaction

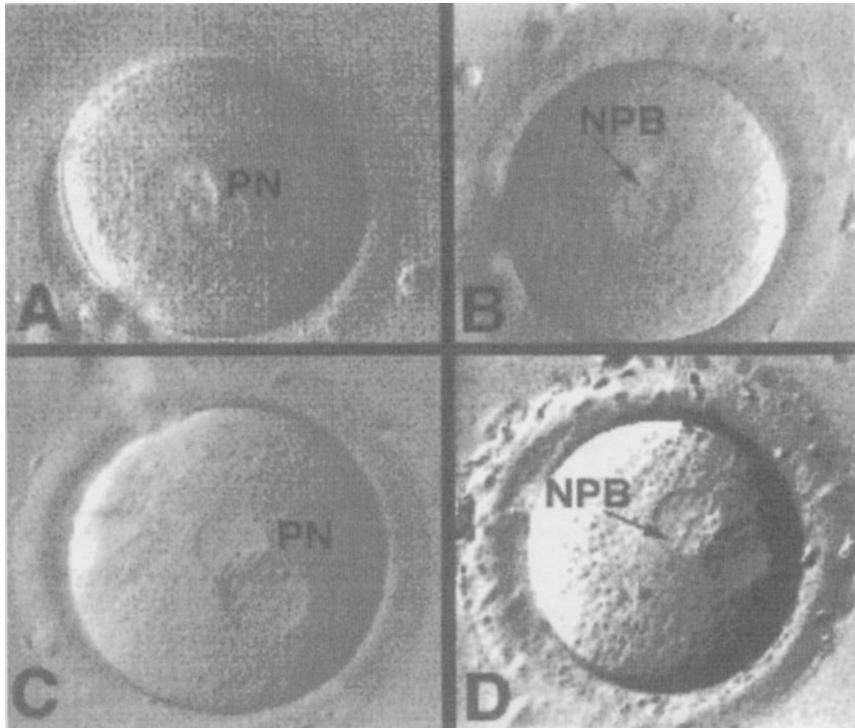


FIGURE 13.4. Fertilized oocytes showing NPB patterns and the halo. A: NPBs of equal numbers, sizes and aligned in the furrow between the nuclei, good morphology; B: NPBs of equal numbers and sizes with a similar pattern of distribution in the 2 nuclei. This is normal but delayed in the condensation of chromatin onto the spindle. C/D: Oocytes in which there is unequal number, size and distribution of NPB in the 2 nuclei. These are abnormal.

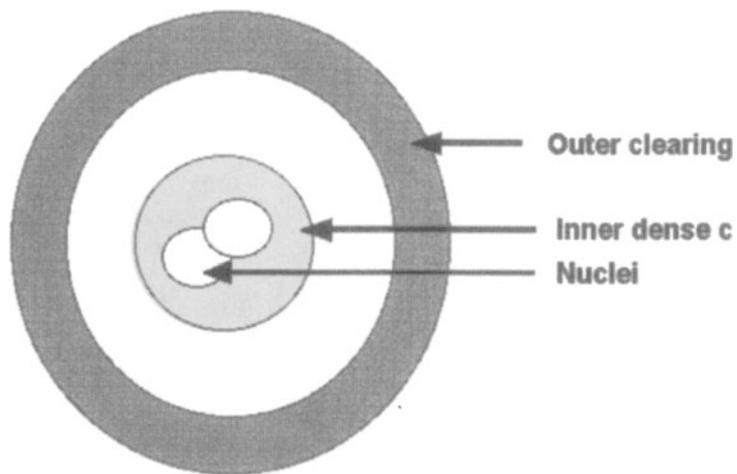
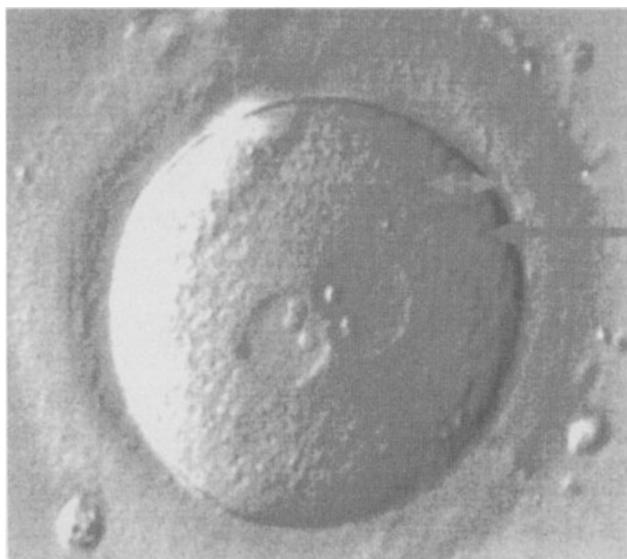


FIGURE 13.5 The cytoplasmic halo or differential cytoplasmic density in a living human fertilized oocyte. There is a clearing in the pericortical region with a dense core in the center.

has not occurred correctly or too early (< 8-cell stage) the embryo may have limited ability to continue development. Early compaction will also result in an embryo that has too few cells and thus allocates all its cells to the outside, resulting in a trophoblastic vesicle in which a functional ICM is absent and therefore the embryo has no capacity to develop into a fetus (Johnson and Ziomek, 1981). All blastomeres contain refractile bodies, which begin to fuse after compaction (Calarco and Brown, 1969). This fusion is time dependent and can even occur in isolated blastomeres. The fusion begins to form the blastocoel.

EXTENDED CULTURE AND BLASTOCYST TRANSFER

The use of extended culture for embryo selection coupled with blastocyst transfer has been proposed as a means of decreasing the number of embryos transferred in IVF cycles without necessarily compromising the probability of implantation, and in some views, actually increasing the probability of implantation. In theory it is generally assumed that an embryo reaching the blastocyst stage has been through embryonic gene activation and by attainment of this stage should have increased developmental potential. The clinical use of blastocyst transfer has indeed shown higher implantation rates compared with day 3 results in selected groups of patients (Gardner and Lane, 1997; Gardner, Vella et al., 1998; Huisman et al., 2000; Milki et al., 2000; Scott et al., 2000; Kovacic et al., 2002) including older ones (Shapiro et al., 2002). In these studies, only patients with an adequate cohort of embryos with normal day 1, 2 or 3 morphology were retained in culture and transferred on day 5. to date, only a few randomized, prospective studies have been performed. However, the findings for good prognosis patients demonstrate that pregnancy rates are equivalent for day 3 and day 5 transfers (Gardner, et al.; 1998; Coskun et al., 2000).

At present, it is widely assumed in clinical IVF, that if an embryo has not formed a blastocyst in vitro, it would have had no potential in vivo if transferred earlier. However, there have been no large-scale studies that incorporate prospective randomized trials to show this is indeed the case. Further, it is evident from the culture data that media in current use may not be ideal or even adequate for blastocyst development for all embryos, or indeed for all patients. It seems likely that day 5 transfers may select for certain type of embryos, rather than those with although viable, do not form

blastocysts but are not necessarily destined for failure. Another aspect of blastocyst culture is the fact that it does not necessarily eliminate aneuploid embryos because embryos with both numerical chromosome abnormalities and mosaicism form morphologically normal appearing blastocysts (Magli et al., 2000; Sandalinas et al., 2001). Further, fertilized oocytes with unequal and mal-aligned NPB, which have been shown to have reduced or no implantation potential and have a higher rate of aneuploidy in select groups of patients, also grow to the blastocyst stage.

In two reports where all patients in the program had blastocyst transfer on day 5 (Marek et al., 1999; Wilson et al., 2002) there was a very low overall implantation rate. In one study (Marek et al., 1999) there was a slight improvement in implantation rate compared to previous day 3 transfer outcome data in the same group of patients (in the same clinic: 29.5 to 38.9% in women under 35); However, this apparent improvement came with the transfer of more than 2 blastocysts, which means that more than 60% of blastocysts failed to implant. In another study where all patients were transferred on day 5, there was an increase in implantation rate compared with previous day 3 rates, from 30 to 50% in the younger age groups, but little improvement in women over 35 years (Wilson et al., 2002). Again, the implantation rates indicate that greater than 50% of transferred blastocysts failed to implant. This could be due to the aneuploidies that exist in many blastocysts (Magli et al., 2000; Sandalinas et al., 2001) and the fact that Z3 fertilized oocytes can form blastocysts.

When the blastocoel is formed during cavitation, the outside cells are committed to form the trophectoderm. Trophectoderm cells should be small, evenly sized cells on the periphery of the embryo, indicating the proliferation of this cellular monolayer. At this point the blastocoel should centrally located. As the blastocyst expands the trophectoderm cells transform into giant-cells and begin to elongate, more in the mural than polar region. At this stage of preimplantation embryogenesis the ICM should become apparent at one edge as a tightly compacted mass of equally sized cells. At the junction between the polar and mural trophectoderm, processes or finger like projections (filopodia) that extend from the migrating polar cells to the ICM can often be seen (Flemming et al., 1984; Gardner, 2000). These usually disappear as further blastocyst expansion occurs, and their persistence in the fully expanded blastocyst seems to have a negative impact on implantation (Scott, unpublished). The above features have been included in a number of blastocyst scoring schemes (Balaban et al., 2000; Dokras et al., 1993; Gardner et al., 2000; Gianaroli et al., 2000). Blastocysts that have normal competence show little expansion and thus a small blastocoel, or can have no discernable inner-cell mass through expansion to the fully expanded stage. The current scoring systems lack detail regarding the rapid progression of blastocyst

formation from early stages to full expansion, but all require that an ideal blastocyst have a blastocoel, a band of evenly spaced equal sized mononucleated cells in the trophectoderm, and an obvious ICM that is compact and comprised of equal sized cells at approximately 154 h post-hCG, or 112 to 114 hours post insemination. Blastocysts with finger like projections stretching across the blastocoel from the trophectoderm to the ICM show a failure of full migration of the polar cells to the mural region have lowered implantation rates. However, since there is a progression in this development, many of these can/will retract with time (Scott, 2001b).

Although blastocyst transfer has a place in the IVF lab, it is not the answer to the low implantation rates currently seen. Blastocysts can form from grossly abnormal oocytes, fertilized oocytes and embryos and from embryos that are aneuploid. Having a total picture of the embryo, from fertilized oocyte to blastocyst, and being able to assess its viability, just as with earlier stages of embryogenesis, is still of prime importance because sub-optimal culture or laboratory conditions and intrinsically flawed oocytes or early embryos may still develop to the blastocyst stage, may appear morphologically normal but are developmentally unviable with respect to the purpose of clinical IVF, the generation of a normal child.

CONCLUDING COMMENTS

Due to the complex and dynamic process from oocyte development, through fertilization, early cleavage and blastocyst formation there is no one parameter currently available that can predict the viability of an embryo after transfer. The selection of the most viable embryo for replacement requires a sequential scoring system, such as the one shown in Table 13.1. The selection should start with the follicle. Careful attention should be paid to its growth pattern and correlated rise of estradiol as the granulosa cells proliferate and differentiate during the follicular phase of the cycle. The state of vascularization should be measured. All facilities have ultrasound machines and this is a feasible operation and one that could maximize outcome (see Gregory, this volume). The exact time of the hCG injection must be noted, as this is the time point from which all embryo development should be timed.

The second stage of selection of the oocyte can be done at this point at the MII phase during ICSI cases, where the oocytes are stripped of their cumulus mass and can be visualized. Oocytes with large abnormal polar bodies should not be used as they display the results of a breakdown of a fundamental aspect of development. Those with fragmenting polar bodies should be noted and used only if they meet all other selection criteria during development.

After fertilization the fertilized oocytes can be scored for monospermic fertilization and 2 polar bodies. Any fertilized oocytes not adhering to this

normal developmental progression. The halo effect and the fertilized oocyte score (the NPB patterns) is the next stage of competence assessment with all abnormal fertilized oocytes again being set aside, since they have little implantation potential and have a higher incidence of chromosomal abnormalities.

Early entry into the first mitotic division with the resultant early formation of a 2-cell embryo by 22-24 h post fertilization (62-68 h post-hCG) will further select those normal fertilized oocytes with increased developmental potential. The morphology of the day 3 embryos, stage appropriate cell number, and degree of fragmentation and blastomere regularity are then added. If no more than 2 or 3 embryos pass these selection criteria, in my opinion, embryo transfer should be performed by day 3. Since extended culture media are not yet optimized, up to 60% of blastocysts fail to implant, and many embryos with known abnormalities can reach the blastocyst stage, it is unlikely that anything could be gained from continuing to culture a few embryos that have passed all selection criteria up to the 8-cell stage. In natural cycles, 50% of embryos grown entirely in vitro have also been shown to fail to reach the blastocyst stage and of these only 40% implanted (Vlaisavljevic et al., 2001). If only 40-60% of embryos reaching the blastocyst stage in stimulated cycles are implanting, the benefit of extended culture when there is not a large cohort of like morphology day 3 embryos is probably minimal. If, however, there is a large cohort of embryos in which all have passed these selection criteria, a day 5 transfer may be warranted. In this way the most viable of the cohort can be selected.

In conclusion, the process of oocyte and embryo development is complex and no one scoring system can adequately select the one TOP quality embryo that has the highest potential to implant. Scoring and embryo selection needs to be done with biology in mind at every step in the process. It needs to be optimized for each patient and cohort of embryos. As more of the control mechanisms in development are elucidated and the media improved it may become possible to select embryos using biochemical markers. Until then, observation and application of biological principles will aid in selecting those embryos with the highest potential to implant and develop to term.

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CHAPTER FOURTEEN

THE ENIGMA OF FRAGMENTATION IN EARLY HUMAN EMBRYOS: POSSIBLE CAUSES AND CLINICAL RELEVANCE

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INTRODUCTION

Blastomere fragmentation is one of the most enigmatic aspects of early human embryogenesis. Within cohorts of embryos maintained under identical conditions, including those cultured in the same dish, fragmentation affects none, some or all embryos during the early cleavage stages. The importance of fragmentation in clinical IVF is related to several questions: First, is this phenomenon indicative of reduced competence or a premorbid condition that argues against transfer to the patient or cryopreservation for future attempts at pregnancy? Second, is fragmentation a normal process in early human development or an in vitro artifact, perhaps associated with suboptimal culture media and conditions, which have yet to be identified? Three, what is the cellular or molecular basis of fragmentation, and do different patterns and extents of fragmentation have a common or different etiology. Virtually all empirically based systems currently used to assess performance in vitro and select human embryos for transfer include some form of fragmentation characterization. For some schemes, the degree of fragmentation is a subjective estimate based on the relative size of the fragment population observed by light microscopy (slight-to-extensive, e.g. Puissant et al, 1987; Giorgetto et al, 1995; Hoover et al, 1995). Depending upon protocols used in clinical IVF laboratories, numerical (e.g., 1-to-4) or letter grades (e.g., A-to-D) may be assigned to represent the apparent degree of fragmentation, which is often determined at a single inspection during cleavage. Other schemes have been stated to be objective, with the degree of fragmentation expressed

in percentiles such as less than 10% or greater than 25% (Morgan et al, 1995; Alikani et al, 1999). However, the biometric methods or algorithms used, if any, in objective analyses are rarely described, and a statistical basis for quantitation of fragment numbers or volume is usually not demonstrated. Therefore, whether static measurements of these parameters are accurate and quantitative can be difficult to determine independently from most published studies.

The issue of accurate quantitation is particularly evident for some proportion of affected embryos where a plieomorphic and densely packed population of fragments often occurs at one or more locations, making the identification of all individual fragments difficult and determinations of actual volume problematic, even with digital imaging and computerized processing. Another issue is one of spatial and temporal origins, namely, whether fragments arise from multiple blastomeres or from the destruction of an entire blastomere(s), and whether fragments observed at the 4- or 8-cell stage arise abruptly or accumulate progressively with cell division. Recognition of the cellular origins of fragments may be both biologically and clinically relevant in assessing developmental potential, as the loss of blastomeres could indicate underlying cellular defects leading to self-destruction by cell death pathways, while the occurrence of fragments in otherwise intact embryos may or may not have adverse developmental consequences. The actual number of intact blastomeres in fragmented embryos can be difficult to assess beyond the 6-to-8-cell stage, and in both objective and subjective schemes, some observers may consider relatively large anucleate structures to be fragments. Such assumptions can be incorrect and confound conclusions as to whether an embryo is actually stage-appropriate because the presence of a nucleus is cell cycle related, and increased asynchrony in the timing of the cell cycle between blastomeres is evident as cleavage progresses to the morula stage.

DOES FRAGMENTATION HAVE AN IATROGENIC ORIGIN?

It has been proposed that fragmentation in early human embryos is an apoptotic process that involves activation of programmed cell death (PCD) pathways in some or all blastomeres, possibly as a consequence of putative cytoplasmic, molecular, or chromosomal disorders. If correct, fragmentation may represent a fundamental developmental capacity of the human embryo to 'self-correct' by eliminating defective cells during early development, or to 'self-destruct' if the presumed developmentally lethal defects involve most or all blastomeres. PCD and apoptosis are normal processes in invertebrates and vertebrates that are essential in the development and morphogenesis of tissues and organs, but can also be induced *in vitro* in certain cell lines by changes in media composition (e.g., serum, amino acid or growth factor deprivation) or

culture conditions (e.g., hypoxia). Hypoxia has also been proposed to be a potent inducer of apoptosis in the hindbrain and otic vesicles of 11 day old rat embryos, suggesting that hypoxia is normally required to activate apoptosis *in vivo* in order for proper morphological development to occur (Chen et al, 1999). Experimental findings from defined cell lines raise the possibility that some embryos, or blastomeres within embryos, may have unique and adverse responses to certain *in vitro* conditions that could activate PCD pathways. This may be particularly relevant in instances where heavily fragmented embryos found to be chromosomally normal occur in the same culture dish with intact and developmentally progressive siblings. The possibility that fragmentation may have an iatrogenic origin associated with currently undefined differences between embryos within cohorts, differences which may make some uniquely susceptible to particular *in vitro* conditions, raises the question of whether this activity occurs during early embryogenesis *in vivo*.

The available literature on early human embryo development *in vivo* is limited to a very few reports, such as the pioneering studies of Hertig et al (1954; reviewed by Hertig and Rock, 1973), who examined preimplantation stage embryos found in hysterectomy specimens (fallopian tubes and uterus), and from the work of Buster et al (1985), who used artificial insemination and uterine lavage on day 5 to obtain embryos from stimulated ovum donors for infertility treatment. Hertig et al (1954) observed a high proportion of abnormal appearing, developmentally arrested, and deteriorating embryos in their collection of paraffin sectioned material. However, it is uncertain whether fragmentation as currently observed in embryos maintained *in vitro* is comparable to the *in vivo* situation. Perhaps the best evidence for fragmentation *in vivo* comes from the study of Buster et al (1985), where embryos characterized as poor quality or stage-inappropriate, contained degenerate blastomeres and extracellular fragment-like structures. Such embryos occurred at relatively high frequency in the presence of normal appearing blastocysts capable of development to birth after transfer to recipients. Of particular interest with respect to development and fragmentation was the occurrence of a tubal pregnancy that resulted form a 12-cell embryo, which contained both normal and degenerate blastomeres on day 5. It is unknown whether the patterns and types of *in vitro* embryo fragmentation discussed below have counterparts *in vivo* or occur at similar frequencies. Evidence for an iatrogenic phenomenon would be suggested if improvements in the design of media for early human embryo culture clearly resulted in a very significant decline in fragmentation frequency or its virtual absence. Although claims of reduced fragmentation rates have been made for different culture media suggested to be optimized for the preimplantation stage human embryo (for review, see Biggers, this volume), confirmation will

require well designed prospective, randomized trials as described by Kolibianakis and Devroey (2002)

DO DIFFERENT PATTERNS OF FRAGMENTATION HAVE SPECIFIC TEMPORAL AND SPATIAL CHARACTERISTICS?

Although most reports of fragmentation have focused on the early cleavage stages, fragmentation can also occur at both oocyte and pronuclear stages, albeit at very low frequency. For example, the fragmented germinal vesicle (GV) stage oocyte in figure 14.1A1 is shown as observed at retrieval. However, despite this clearly abnormal morphology, the oocyte was inseminated for experimental purposes and within 1 hour, was penetrated by a single spermatozoon (arrow S, Fig. 14.1A3). Regardless of whether meiotic maturation occurs *in vivo* or *in vitro*, fragmentation between the GV and metaphase II (II) stages seems to be exceeding rare (Van Blerkom and Davis, 1998). In our documented experience (Van Blerkom, unpublished), <000.5% of MII (151/35,400) and only a handful of GV stage oocytes have been classified as fragmented at retrieval. Figure 14.1B1 is another example of a rare fragmented oocyte, and in the few highly fragmented post GV stage oocytes we have examined, few had DNA detectable after staining with fluorescent probes (Fig. 14.1B2). All of the fragmented GV and post GVB oocytes observed at retrieval remained unchanged with respect to fragment number, intactness and position during 3-to-4 days of culture. The images shown in figures 14.1C1-to-1C5 are examples of pronuclear embryos that exhibit different degrees of fragmentation, which in these cases, was first detected between 12 and 16 hours after conventional IVF. In our experience, <1.5% of pronuclear embryos (491/35,400) exhibit fragmentation of the type shown in these figures, with only a handful showing complete fragmentation as presented in figure 14.1C5. This pattern of fragmentation at the 1-cell stage is always associated with developmental lethality. However, after the initial fragmentation event occurs, sequential microscopic inspections of affected embryos indicate that the apparent number of fragments and their location tend to remain largely unchanged during subsequent culture. Not all instances of fragmentation at the pronuclear stage are lethal and as described below, Antczak and Van Blerkom (1999 reported certain fragmentation phenotypes where most of the affected embryos continued to develop *in vitro* and were shown to be competent by virtue of their ability to develop to birth after uterine transfer.

Several studies have asked whether (a) distinctly different patterns of fragmentation occur during the early cleavage stages, and (b) whether they have specific spatial or temporal characteristics that can be related to subsequent performance *in vitro* or outcome *in vivo*. Alikani et al (1999) first

described a fragmentation classification system that has been widely adopted for day 3 embryos (Boiso, 2002). Five distinct patterns of fragmentation were identified and the severity for affected embryos was reported in percentile form, representing the volume of the perivitelline space and/or cleavage cavity occupied by the fragments. Based on the images descriptions presented, the following fragmentation patterns were observed: Type I, 0%-5%, minimal in volume and usually found as a cluster associated with one blastomere; Type II, relatively larger fragments localized as a cluster within the perivitelline space; Type III, small, scattered fragments within the cleavage cavity or perivitelline space; Type IV, large fragments with some of blastomere size distributed randomly throughout the embryo which typically has uneven cells; Type V, fragments which appeared necrotic. The extent of fragmentation for individual embryos in each group was reported in five percentiles of increasing severity; 0%-5%, 6%-15%, 16%-25%, 26%-35%, and $\geq 35\%$. The actual method of fragment volume quantitation indicated as capable of providing such specific percent ranges was not described, nor were mathematical transformations, if any, presented that may be required to measure volume in a plieomorphic population fragments. However, outcome findings involving transfer of unmanipulated embryos indicated that certain patterns of fragmentation, even when detected at high levels, were not detrimental to implantation potential or the capacity of implanted embryos to develop to term. For example, the occurrence of small, scattered fragments (Type III) had no appreciable effect on implantation frequency even at a relatively high percentile. Alikani et al (1999) suggested that this pattern may not be indicative of anomalous development and in these instances, fragments could arise during normal cell divisions. Type II embryos contained fewer cells than appropriate for days 2 and 3, which may result from the complete fragmentation of one or more blastomeres. However, an implantation rate of 34% was reported for these embryos despite the presence of highly localized and plieomorphic fragment clusters. The finding that developmental competence is not always abolished despite relatively severe fragmentation and apparent cellular loss suggests that for certain embryos, an inherent ability exists to maintain viability even if putative defects or dysfunction effect particular blastomeres. Alikani et al (1999) also reported that the mechanical removal of fragments significantly improved implantation rates and suggested that competence in severely fragmented embryos may result from (a) restoration of normal, stage-specific spatial relationships between blastomeres required for compaction, or (b) the elimination of potentially toxic influences that may be released during the degeneration of extracellular fragments.

The notion that the presence of fragments between blastomeres may hinder normal spatial interactions required for compaction was suggested in a

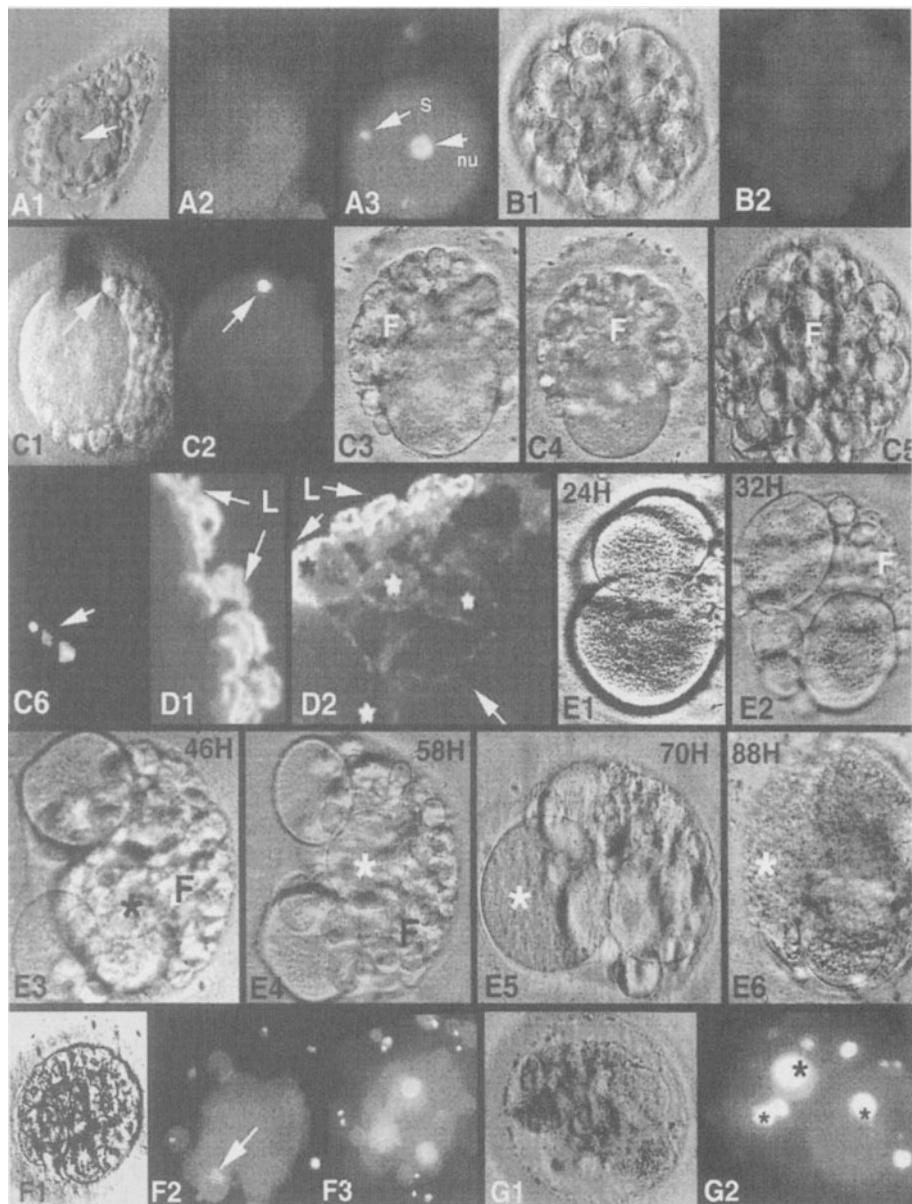
follow-up study by Alikani et al (2000) that examined the capacity of embryos with various cleavage anomalies to compact, cavitate and develop to the blastocyst. For example, rates of blastocyst formation were no different between undisturbed embryos with Type I (38.6%), Type II (32.9%) or Type III (32.4%) fragmentation. Type IV embryos showed a reduced capacity (14.7%) to develop to this stage. Regardless of fragmentation type, normal blastocyst formation decreased significantly (33.3% at 0-15% fragmentation) as the apparent severity of fragmentation increased (16.5% at >15% fragmentation). Light microscopic inspection of fragmented embryos for compaction-related activities suggested that regional or discontinuous compaction may result from the prevention of a close association of opposed blastomere plasma membranes during late cleavage. With continued development, some affected embryos contained excluded cells in the blastocyst cavity, an activity that was suggested to be associated with reduced competence. These findings tend to lend support to the practice of mechanical fragment extraction in order to improve post cleavage performance and developmental competence.

Antczak and Van Blerkom (1999) examined spatial and temporal aspects of fragmentation in early human embryos and described the following four distinct patterns of fragmentation from the pronuclear through the 8-cell stages, some of which appear similar to the phenotypes reported by Alikani et al (1999): Type I, a monolayer-like carpet of very small fragments that coated only a 'modest' portion of the cell surface with either no apparent reduction in cell size, or one which was relatively minor but evident at the light microscopic level; Type II, multiple layers of fragments, generally involving a considerable portion of the cell surface and accompanied by a significant reduction in the size of the affected cell; Type III, the complete disintegration of a blastomere(s) in embryos showing no fragmentation on previous inspections; Type IV, occasional fragments of varying size scattered over several blastomeres in otherwise normal-appearing, developmentally progressive embryos. The finding that certain regulatory proteins detected in cortical domains (Antczak and Van Blerkom, 1997), such as leptin (L, leptin immunofluorescence, Figs, 1D1,D2) were also present in fragments suggested the possibility that developmental competence could be compromised if extensive elimination of regulatory proteins occurred (Antczak and Van Blerkom, 1999). However, it was unclear from this light and immunofluorescence microscopic study whether specific regulatory protein-containing fragments were actually detached and therefore extracellular, or might still be associated with the underlying blastomere. In some instances, an apparent continuity between the interior of the fragments and cortical ooplasm was suggested. This issue was particularly difficult to resolve for Type I (e.g., 14.1D1) and Type II fragments when they occurred in overlapping columns

(e.g., asterisks, Fig. 14.1D2). However, fine structural studies described below confirmed the impression of continuity between fragments and between apparently detached fragments and the underlying blastomere, especially for those, which occurred in columns.

With respect to performance in vitro, Antczak and Van Blerkom (1999) reported that the presence of Type I fragmentation at the 1-cell stage was largely associated with continued cell division (87%, 97/111) while all those showing the Type II pattern (42/42) remained undivided. At the 2-cell stage, some 38% of embryos in which the Type III pattern resulting from single blastomere fragmentation (80/211) showed no further division while 62% (131/211) underwent at least one additional normal cleavage division. Similar findings were obtained at subsequent stages of cleavage and led to a general conclusion that the earlier in development that Types II and III fragmentation occurred, the more likely it was that developmental competence was compromised. However, by the 8-cell stage, blastomere loss by fragmentation or the occurrence of intact blastomeres with nascent fragment clusters did not preclude development to the expanded and hatched blastocyst stage. Antczak and Van Blerkom (1999) reported that on day 3 (8-to-10 cell), 68% (1185/1743) of stage-appropriate embryos could be classified as Type IV. However, outcome results showed no apparent adverse developmental effects (pregnancy rate = 45%) when compared with transfer cohorts involving intact embryos at the same stage (pregnancy rate = 43%). For day 3 transfers involving only embryos with Type III fragmentation at the 4-cell stage, an observed pregnancy rate of 31% suggested that loss of a viable blastomere by fragmentation may be associated with reduced competence for some embryos, but for others, viability is clearly retained. This notion was supported by the finding that approximately 80% of cleavage stage embryos with Types II or III fragmentation continued to divide beyond the 4-cell stage with

FIGURE 14.1. Figures A-to-E6 are representative of images of different patterns and extents of fragmentation observed in germinal vesicle (GV: A1-A3), post germinal vesicle breakdown (B1, 2) stage oocytes, and pronuclear- (C1-C6) and cleavage-stage human embryos (D1-E6). Figures D1 and D2 are immunofluorescence images of leptin (L) distribution in the apical cytoplasm of fragments and fragment columns (asterisk, Fig. D2). Figures E1-E6 are selected images of fragmentation in a 2-cell embryo taken at timed intervals between 24 (E1) and 88 (E6) hours of culture. The asterisk in figures E3 to E6 indicate the progressive swelling of a fragment followed by lysis. Figures F1-F3 show TUNEL-staining of a fragmented cleavage stage embryo (Fig. F) in which DNA fragmentation was detected in the polar body (arrow, F2). Nuclear staining was detected after exposure to DNase (Fig. F3). Intense TUNEL positive DNA fluorescence in a cleavage stage embryo that lysed after thawing (Fig. G1) is indicated by asterisks in figure G2.



approximately 50% developing into normal appearing blastocysts on day 6. In comparison to blastocysts that developed from intact cleavage stage embryos, blastocysts from 4-cell embryos with type III fragmentation reached a comparable stage with a delay of about 12-to-24 hours.

DO REVERSIBLE AND IRREVERSIBLE FORMS OF FRAGMENTATION OOCUR?

The above findings demonstrate the complexities of interpreting fragmentation with respect to origin(s) and effects on competence. What is clear from both the Alikani et al (1999, 2001) and Antczak and Van Blerkom (1999) studies is that even when the extent of fragmentation appears severe and cellular loss has occurred, competence is often unaffected. Spatial and temporal characterizations of fragmentation have resulted in classification systems that should be applied in clinical IVF, as they may well represent important criteria for assessing relative competence when fragmentation involves a significant proportion of embryos within cohorts. However, these and other fragmentation schemes are largely derived from one or two static inspections timed for operator convenience rather than to the cell cycle. As a result, whether fragmentation is abrupt or progressive is difficult to determined from limited examination.

Our initial studies of fragmentation involved sequential photomicroscopic documentation of embryos taken at 8-to-12 hour intervals. For example, the representative images shown in figures 14.1E1-to-1E6 (hours post-insemination are presented in the upper portion of each figure) indicate little change in the apparent density and distribution of fragments in an embryo which had arrested cell division at the 2-cell stage. However, over time, some fragments underwent swelling (asterisk, Figs. 14.1E3-1E5) and eventual lysis (asterisk, Fig. 14.1E6). Other fragmented embryos within the same cohort(s) progressed no further than the 4-to-6-cell stage but seemed to remain largely unchanged during an additional 3 or 4 days of culture. For example, the embryos shown in figures 14.2Q and R were photographed on day 4 and 5, respectively, after fragmentation was first detected at the 4-cell stage on day 2. Sequential inspection of embryos at intervals of 8-to-10 hours also revealed a surprising phenotype where fragments present at the 2-to-4 cell stage were largely undetectable at the 8-to-10 cell stage, and no residual cellular debris was apparent in the perivitelline space. At transfer on day 3 to 3.5 (8-to-12 cell stage), these embryos appeared normal and stage appropriate, and competence was demonstrated by term pregnancy rates equivalent to those obtained from embryos that were intact throughout culture. We have used time lapse videomicroscopy to study this unexpected behavior as it may relate to etiology of fragmentation, competence, and influence on embryo

classification/selection schemes that use static assessments. In developmental terms, this activity raises the question of whether a capacity exists for certain cleavage stage embryos to undergo 'self-corrective' measures in order to eliminate potential impediments to normal development. A retrospective assessment of notations and images taken from 6800 embryos with Type II fragmentation (according to Antczak and Van Blerkom, 1999) at the 4-cell stage (day 2) showed that fragments were largely undetectable at the 8-to-12 cell stage (day 3-3.5) in 2587 (38%) stage-appropriate embryos. Typically, this data was obtained from two or three embryo inspections.

This unusual and not uncommon behavior led to a multiyear time-lapse study of embryo performance in vitro from the pronuclear through the cleavage stages. To date, we have recorded the development of 91 human embryos (51 donated monospermic, 40 dispermic) which showed relatively heavy fragmentation during the early cleavage stages, but which were stage-appropriate and largely normal in appearance on days 3.0-3.5 (Van Blerkom et al, 2001). Temporal aspects of fragmentation determined by time-lapse imaging demonstrated that the initial fragmentation event occurred over a 30-minute period in approximately 90% of embryos. This activity was embryo-specific, with Type I (21%), Type II (47%), Type III (2%) or Type IV (30%) patterns (Antczak and Van Blerkom, 1999) exhibited at the 2-to-4 cell stage. For approximately 10% of the embryos examined, fragmentation Types I and II seemed to be episodic, with fragments occurring both at the 2- and 4 cell stages. Embryos with Type II fragmentation were especially informative because not only is this pattern common, but with current embryo classification systems, we considered it to be the most problematic in assessments of viability and competence.

Time lapse analysis was extremely useful in following the genesis and fate of fragments, and by continuous imaging, enabled documentation of when and which fragments either arose, changed position or disappeared during culture. The representative timed images shown in figures 14.2A-P present two different degrees of Type II fragmentation, slight (2A-H) and moderate (2I-P). Embryos exhibiting this activity were examined in detail from the pronuclear through the 4- (Figs. 14.2A-H) and 8-cell stage (Figs. 14.2I-P). In these instances, individual fragments or columns of fragments seemed to disappear by resorption into the underlying blastomere. Although not evident in these static images, resorption was suggested by turbulence in the region of the cytoplasm directly subjacent to a particular fragment as if the contents of the fragment were either expelled or withdrawn into the blastomere. Typically, the disappearance of fragments was associated with this focal cytoplasmic activity and occurred within 5 minutes or less. For some embryos, virtually all of the apparent extracellular fragments disappeared over a 2-to-12 hour period. The finding that relatively large fragments can be

resorbed into the underlying blastomere has been recently confirmed in a high-resolution time lapse imaging study of cleavage stage human embryos by Hardarson et al (2002). For some Type II embryos, we observed that a variable proportion of apparent fragments were resorbed. Within the same

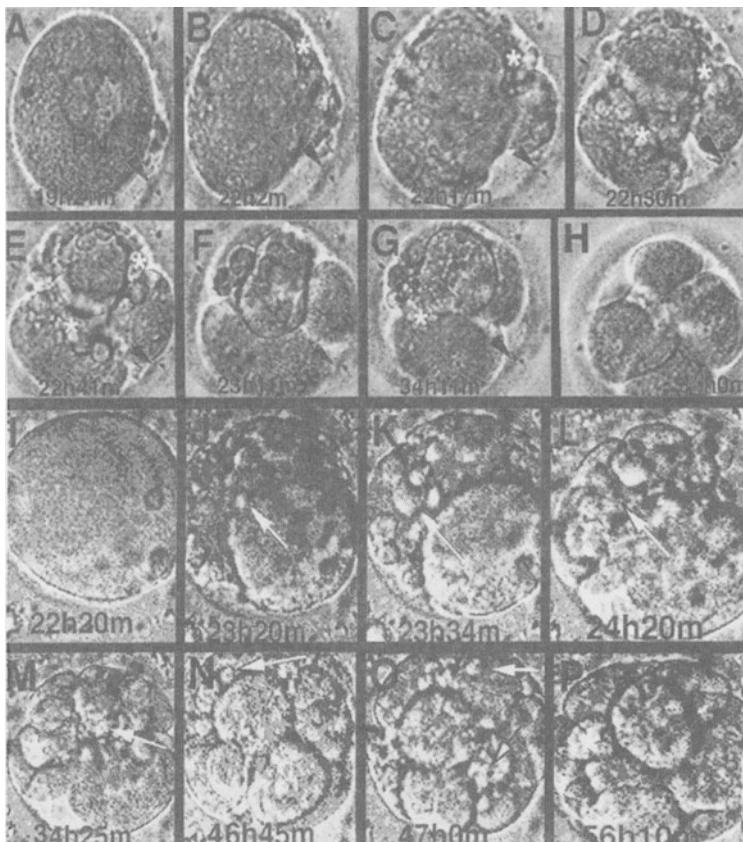


FIGURE 14.2. Selected light microscopic images from a time-lapse sequence of in vitro development of two human embryos from the pronuclear to the 4-cell stage (A-H) and from syngamy to the 8-cell stage (I-P). These representative embryos developed Type II fragments during the first cell division but were largely intact and stage appropriate at transfer on day 3-3.5 post fertilization (time in hours (h) and minutes (m) after fertilization is given in the lower portion of each panel). (From Van Blerkom et al, 2001).

embryos however, others fragments remained intact for prolonged periods of time, but were often observed to undergo an abrupt swelling (in minutes) and lysis (in seconds). Fragment lysis occurred throughout cleavage and was a fragment-specific activity that seemed to be unrelated to cell division, position, or other morphodynamic activities occurring within the embryo (Van Blerkom et al, 2001).

Lysis and resorption may reflect fundamental differences between fragment-like structures. Van Blerkom et al (2001) used the term pseudo-fragments to describe fragment-like protrusions of the blastomere plasma membrane that varied in number and size. In the case of lysed fragments, detailed analysis of time-lapse images demonstrated that they were indeed detached structures while resorbed structures, present individually or in columns, remained firmly associated with the underlying blastomere. Serial section reconstruction of fine structural images of embryos whose fragmentation activity was documented by time lapse microscopy showed that some apparently detached fragments, as well as those occurring in extended columns, retained cytoplasmic continuity both with one and other and with the underlying blastomere. Examples of such continuity are shown in the column of fragments depicted by an asterisk in figures 14.4A and B (for details, see Van Blerkom et al, 2001). These findings provide a structural basis for the resorption of surface protrusions that would likely be classified as extracellular fragments by conventional light microscopy (Hararson et al, 2002). One of the most unexpected and remarkable activities we recorded was observed in 2-cell embryos which appeared to undergo a sudden and complete fragmentation of one (embryo *ii*, Fig. 14. 3C) or both blastomeres (embryo *i*, Fig. 14.3C), or were classified as severe Type II fragmentation (embryo *iii* Fig.14.3C). In some exceptional instances, apparently intact cellular structures (1,2,3, embryo *i*, Fig. 14.3F) developed from a mass of fragments after approximately 20 hours of culture. In this same representative cohort, some fragmented embryos remained unchanged during culture (e.g., embryo *ii*, Figs. 14.3C-F), while others were progressive and appeared relatively normal and stage appropriate (e.g., embryo *iii*, Fig. 14.3F).

Time-lapse analysis also demonstrated that fragments, either individually or in clusters, could change position in concert with movements or rotations of the associated blastomere (e.g., arrow, Figs. 14.2J-M). In these instances, the apparent disappearance of fragments from the apical or exposed surface of blastomeres could result from internalization into interior spaces within cleavage stage embryos rather than from resorption. Careful inspection of time-lapse images suggested that certain clusters of fragments were not nascent but rather resulted from re-emergence as a particular fragment-associated region of the blastomere(s) surface returned to a subzonal location, which allowed clear visualization of the exposed surface. Electron

microscopic images demonstrated the presence of fragments between blastomeres within the interior of the embryo (e.g., Fig. 14.4B; see Van Blerkom et al, 2001, for details), suggesting that some proportion of fragments may remain within the embryo. However, we reported that embryos with internalized fragments continued to divide and were developmentally competent on day 3 as demonstrated by ongoing pregnancy rates (55%) comparable to those observed with intact embryos (63%).

Collectively, these results indicate the transient nature of ‘fragments’ that occurs in certain common fragmentation phenotypes. Consequently, the time of inspection and the assessment system used could result in different classifications of cleavage stage embryos that have clinical significance with respect to which are selected for transfer or cryopreservation. Outcome results

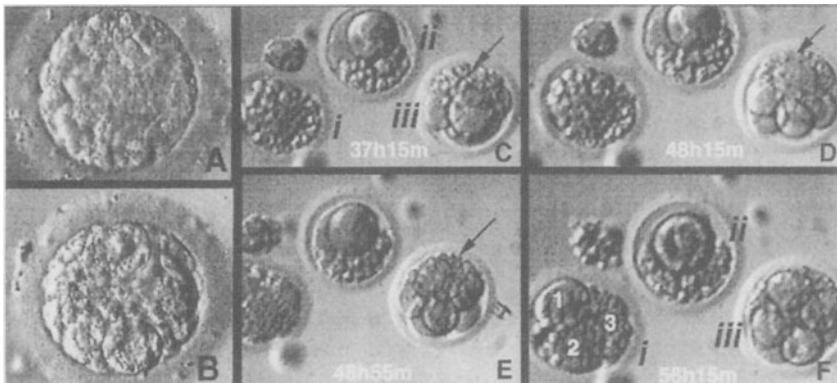


FIGURE 14.3. The embryos shown in figures A and B showed complete fragmentation at the 4-cell stage, but with respect to apparent fragment number and distribution, remained unchanged for 3 and 4 days, respectively, after fragmentation was first detected on day 2 post fertilization. Figures C-E show a rare and remarkable activity displayed by highly fragmented embryos. In this instance, fragmentation at the 2-cell stage ranged from complete to partial in three embryos in the same cohort. However, over time, a largely normal 8-cell embryo developed (embryo iii) while two distinct blastomere-like cells appeared to form from a mass of fragments the first occurred at the 2-cell stage (embryo i). In contrast, embryo ii remained unchanged. (from Van Blerkom et al (2001)

associated with the phenotypes and activities described by Alikani et al (2000) and Van Blerkom et al (2001) indicate the necessity for serial inspections of suspect embryos before a final decision is made concerning their disposition. However, it is also clear that other forms of fragmentation are so extensive

(e.g., Figs. 14.1E1-E5; 3A,B; embryo *ii*, 14. 3C) that no recovery is possible, cell division ceases, or in the extreme example described for embryo *i* in figures 14.3C-F, apparent cellular reformation results in an ‘embryo’ whose developmental competence and normality is suspect. In these cases, transfer or cryopreservation would not be indicated.

POSSIBLE ETIOLOGIES OF FRAGMENTATION IN EARLY HUMAN EMBRYOS

APOPTOTIC MECHANISMS

One view of fragmentation in early human embryos considers this activity to be an apoptotic process resulting from the activation of programmed cell death (PCD) pathways (Jurisicova et al, 1995,1996; Levy et al, 1998). The finding that human oocytes and early embryos express (Brenner et al, 1998; Jurisicova et al, 1998,2003; Warner et al, 1998; Antczak and Van Blerkom, 1999;Liu et al, 2000) some of the proteins involved in apoptosis (e.g., Bax, Bcl2, Bclx, Caspase 2, Hrk) indicates that an apoptotic capacity exists and may become a primary means of cellular elimination at the late morula and blastocyst stages (Hardy, 1999). According to this interpretation, fragmentation may be a process whereby blastomeres with cytoplasmic, chromosomal, or other genetic defects are selectively eliminated during the earliest stages of embryogenesis (Jurisicova et al, 1995). To date, evidence suggestive of apoptosis in early cleavage stage human embryos has come primarily from the expression of apoptotic genes (Liu et al, 2000; Jurisicova et al, 2003) and from the following: (a) fine structural images of affected embryos, (b) the detection of DNA breaks by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL assay), and (c) the occurrence of phosphatidylserine (PS) residues detected with fluorescent-tagged annexinV (AV staining).

FINE STRUCTURAL ANALYSIS OF FRAGMENTED CLEAVAGE-STAGE HUMAN EMBRYOS

Apoptosis as a cause of fragmentation in human embryos was proposed by Jurisicova et al (1995) on the basis of TUNEL analysis and images obtained by scanning (SEM) and transmission electron microscopy (TEM). In this study, spheroidal structures suggested to represent apoptotic bodies were confined to about 25% of the apical surface of an 8-cell embryo imaged by SEM. It should be noted, however, that Antczak and Van Blerkom, (1999), Alikani et al (1999, 2000), and Van Blerkom et al, (2001) reported that living embryos with the same pattern and extent of fragmentation were often stage-

appropriate and developmentally competent. While SEM can provide useful images of the fragment and blastomere surface, TEM offers more definitive information with respect to cellular organization and the occurrence of definitive apoptotic bodies, especially for cells in which apoptosis is known to occur spontaneously or can be induced experimentally. Jurisicova et al (1996) showed an extensively fragmented and arrested embryo that contained approximately 6 normal blastomeres when fixed on day 4 post insemination. While no nucleated cells characteristic of blastomeres are evident in this image, the embryo does contain a plieomorphic population of fragments and electron dense structures suggested to represent apoptotic bodies, elements undergoing secondary necrosis, and cellular cypses containing 'several' intact mitochondria. Although the fragments and putative blastomeres showed clear degenerative changes, the absence or paucity of organelles such as mitochondria was a common feature for most of the cellular structures in this figure.

For the human, the designation of a specific cause of cell or embryo demise can be problematic if only TEM images are used because within the same highly fragmented embryo, fragments and blastomeres can display very different fine structural characteristics. In clinical IVF, protocol may require that fixation for microscopy is delayed in order to assure developmental incompetence by documentation of cell division arrest. Consequently, because fine structural analysis may involve embryos fixed well after fragmentation and cleavage arrest occurred, time-associated degenerative changes can present cytopathologies whose relationship to the initial cause(s) of fragmentation may be problematic with respect to interpretation. We have performed TEM analysis of 30 embryos whose in vitro development had been monitored by time lapse microscopy and which showed Type II fragmentation at the 2- and 4-cell stages (Van Blerkom et al, 2001). Some of embryos were examined at these stages, while others, if progressive, were fixed at the 8- or 10-cell stage. Analysis of serial thin sections demonstrated that the most fragments and fragment-like structures had an intact plasma membrane that enclosed a cytoplasm characterized by a uniform texture and by the virtual absence of organelles such as mitochondria (asterisk, Fig. 14.4A,B). In addition, regions of contact between opposing fragments often showed apparent specialization of the plasma membrane reminiscent of tight junctions (arrow, Fig. 14.4B, and at higher magnification, arrows, Fig. 14.4C). For some severely fragmented embryos that arrested development at the 4-cell stage and were maintained in culture for one or two additional days, degenerative changes such as vacuolation (V, Fig. 14.4F) and aberrant distributions of organelles (asterisk, Fig. 14.4F) were detected. Where fragment lysis had been recorded by time-lapse microscopy, cellular remnants were clearly evident by TEM (LY, Figs. 14.4B, and F).

A fine structural feature common to both Type II and Type III fragmented embryos was the presence of nucleated cellular remnants (N, Fig. 4D) in which the nuclear membrane appeared intact and was enclosed by a scant cytoplasmic remnant surrounded by an apparently discontinuous plasma membrane (Fig. 14.4E, embryo fixed 26 hours after fragmentation). In some organelle containing fragments as well as in all blastomeres examined, including those with evident cytoplasmic pathologies (e.g., Figs. 14.4D and F), mitochondrial fine structure appeared unaltered (M, Fig. 14.4G) with no evidence of swelling, membrane deterioration or reduction in the electron density of the matrix. While TEM can provide important information about the composition and organization of blastomeres and fragments, it remains to be determined whether the presence or absence of organelles and the occurrence of degenerative features in some cells are consistent with apoptotic body generation, as it is known to occur in other somatic cells. For example, the presence of putative tight junctions between fragments and the occurrence of intact nuclei enclosed by a residual cytoplasm that contains normal appearing mitochondria do not seem to be consistent with apoptosis in somatic cells (Floryk and Ucker, 2000). However, there may be significant variability between fragmented embryos imaged by TEM that could be related to the process by which fragmentation occurred. It remains to be determined whether remnant structures derived from blastomeres that underwent apoptosis have a composition and organization that is distinctly different from fragments generated by other processes.

ANNEXIN V STAINING OF FRAGMENTED HUMAN EMBRYOS

Translocation or “membrane flipping” of phosphatidylserine (PS) from the inner to outer leaflets of the plasma membrane is an early and apparently characteristic event of apoptosis and one that is especially evident in cultured cells exposed to apoptosis-inducing agents or conditions (Martin et al, 1995). Externalization of this moiety can be identified by living cells with annexin V (AV) tagged with an appropriate probe such as fluorescein isothiocyanate. However, owing to loss of plasma membrane integrity, cells undergoing other forms of cell death, such as necrosis, may also stain positive because loss of plasma membrane integrity that permits the entrance of AV and labeling of PS on the inner leaflet of the plasma membrane is associated with this form of demise. For apoptosis, the plasma membrane of apoptotic bodies is generally thought to remain intact such that positive AV staining is presumed to be localized to outer leaflet of the plasma membrane (Martin et al, 1995). Externalization of PS provides recognition sites for macrophage attachment leading to phagocytosis of apoptotic bodies. In order to distinguish between apoptosis and other forms of cell death using AV, living cells are exposed to

vital dyes (e.g., DNA specific fluorescent dye propidium iodide, PI, ethidium homodimer, EH, or the cytoplasmic stain trypan blue, TP), which are excluded if the plasma membrane is intact, as in apoptosis, but taken up when cell death occurs by processes that compromise plasma membrane integrity.

In a study by Levy et al (1998), cryopreserved cleavage stage embryos that were either intact or fragmented 24 hours after thawing were fixed, stained with AV and PI, and exposed to TUNEL reagents. All fragmented embryos showed intense AV fluorescence and chromatin localized to the nuclear envelope in intact blastomeres. The detection of TUNEL- positive chromatin in a single, small structure (see discussion of polar bodies, below) suggested that fragmentation was an apoptotic event, possibly resulting from activation of the PCD pathway. Another set of thawed embryos was classified as developmentally arrested owing to their apparent failure to divide after 24 hours of culture. While these intact embryos showed apparently normal nuclear chromatin distribution, intense AV staining of all blastomeres was interpreted as an early stage of apoptosis. For reasons noted below, the fate of these embryos could not be determined after AV staining. In contrast, embryos that resumed cell division *in vitro* were mostly AV negative.

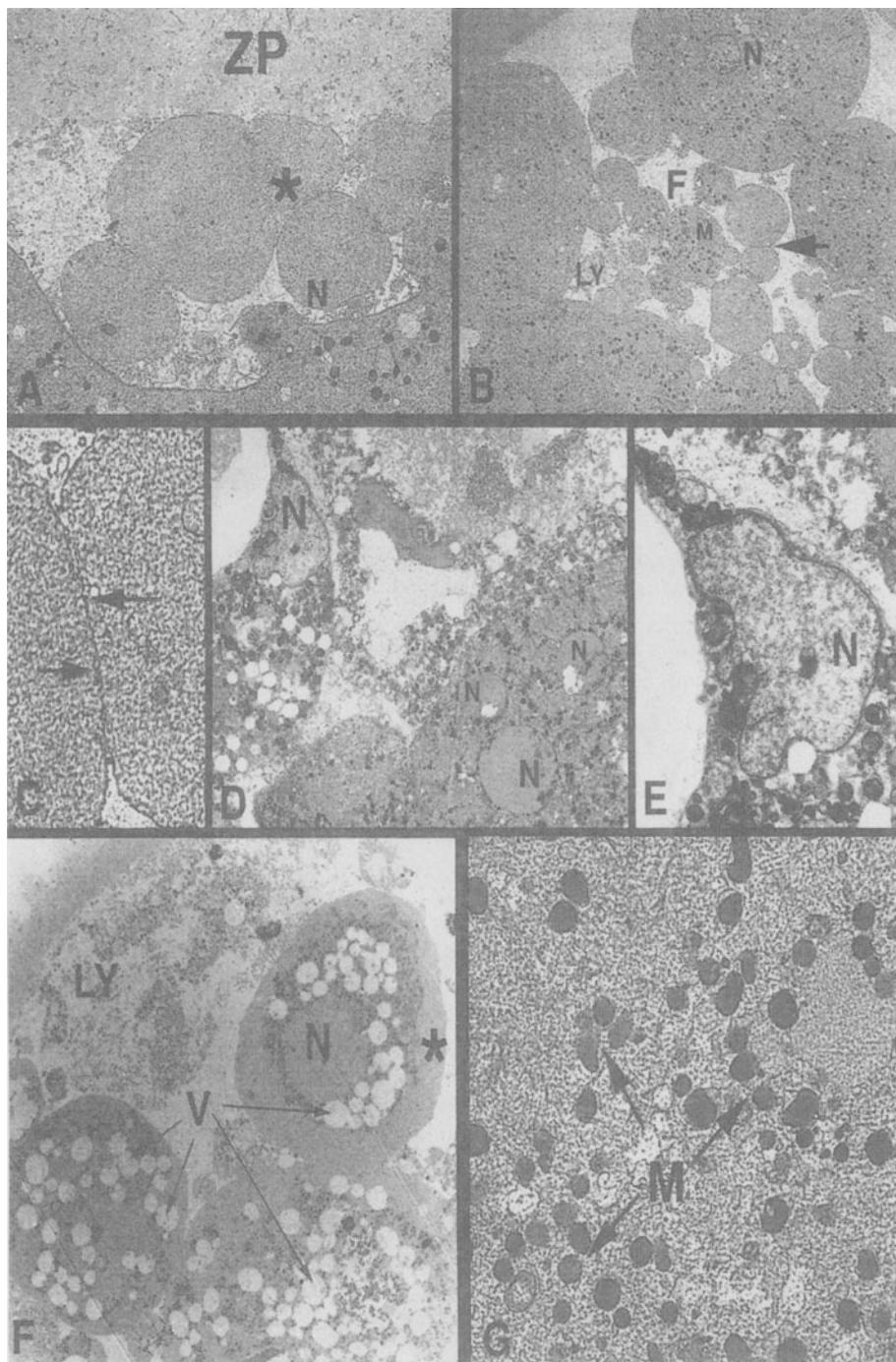
For unfertilized human oocytes, we have previously reported that after several days of culture, most remained intact and were AV-negative. After approximately 5 days of culture, some appeared to have segmented into large cytoplasm-like structures and exhibited patches of AV fluorescence (Fig. 14.5G3) or one intensely fluorescent fragment (asterisk, Fig. 14.5G4; see also Van Blerkom and Davis, 1998). However, AV-positive structures were also found to be TP positive, which suggested loss of plasma membrane integrity could be responsible for staining. In contrast, the first polar bodies in approximately 50% of normal-appearing oocytes examined in this study were both AV (arrow, 14. 5D1) and PI positive (Fig. 14.5D2) when stained within the first 24 hours of culture, suggesting that loss of plasma membrane integrity and AV staining were related. For AV, very similar findings were obtained for living embryos which appeared fragmented during early cleavage but which continued to divide, were stage-appropriate, and largely morphologically normal on day 3.5 (Antczak and Van Blerkom, 1999; Van Blerkom et al 2001). In the nearly 50 embryos examined in these studies, AV staining occurred in a relatively small number of fragments (e.g., arrow, Figs. 14.5E1,E2; asterisk, 14.5F2) that were also either PI or TP positive (asterisk, Fig. 14.5F1). For unfertilized oocytes in which the first polar body divided, AV fluorescence was often confined to only one compartment (arrow, Fig. 14.5D1). The different results obtained by Levy et al (1998) and Van Blerkom et al (2001) may be related to staining protocol in which the former required examination of specimens after fixation in paraformaldehyde or glutaraldehyde prior to exposure to AV and PI. However, all current protocols

for AV use live cells and dyes excluded by intact cells, such as PI, to demonstrate the integrity of the plasma membrane. Van Blerkom et al (2001) restained AV-negative oocytes and embryos (intact and fragmented) after fixation as described by Levy et al (1998) and reported intense plasma membrane AV fluorescence occurred in all cases. It remains to be determined whether AV staining observed in the fixed embryos examined by Levy et al (1998) is related to specific biological process, such as apoptosis. It is also unclear why some developmentally progressive embryos they examined were AV negative after fixation, as this is contrary to our experience with similar material prepared in an identical fashion.

While positive AV staining is an early marker of apoptosis in somatic cells where this form of cell death is known to be occurring or can be induced experimentally, its detection in human embryos should be viewed with caution. For such studies, appropriate controls need to be included to insure that positive staining is associated with PS externalization, rather than nonspecific membrane binding that could result from loss of membrane integrity and internalization of the probe. Our findings indicate that some AV positive fragments do occur in highly fragmented embryos that have arrested division, and in fragmented 2-to-4 cell embryos which were stage-appropriate at transfer (day 3.5). If confirmed, the association between positive TP/PI and AV staining suggests that loss of plasma membrane integrity has occurred. In these instances, AV fluorescence would seem to be unrelated to apoptosis since the corresponding blastomeres remained intact or were demonstrably capable of cell division and typically, adjacent fragments were AV-negative. Additional studies will be required to determine the utility of AV as a marker of embryo developmental competence in clinical IVF.

DNA INTEGRITY IN FRAGMENTED HUMAN EMBRYOS

Cell death resulting from apoptosis is associated with endonuclease-specific DNA breakage into oligonucleosomal-sized fragments. With separation by agarose gel electrophoresis, apoptosis-associated DNA cleavage produces a "ladder-like" distribution of oligonucleosomal fragments of discrete molecular weights, the occurrence of which is considered definitive proof of this form of cell death. The most sensitive gel electrophoretic systems that can detect laddering requires several thousand cells, which makes this analytical approach untenable for the study of single blastomeres or cleavage stage embryos. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) is a widely used histochemical marker to detect DNA breakage during somatic cell apoptosis, especially when the affected cells are known to generate ladders on electrophoretic gels. However, while TUNEL staining alone can detect DNA breaks in single cells, several reports have



shown that this method does not reliably discriminate between necrosis, autolysis and apoptosis (Kressel and Groscurth, 1994; Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp et al, 1995; Frankfurt et al, 1996; Mangili et al, 1999). In addition, nuclei undergoing active transcription can also be TUNEL positive as a result of RNA splicing (Kockx et al, 1998).

In conjunction with putative AV positive staining of fragmented embryos, the occurrence of TUNEL positive fluorescence would seem to present compelling evidence that fragmentation during early human development is an apoptotic process that could be associated with activation of PCD (Levy et al, 1998). Although this view has been widely accepted in clinical IVF, its application to fragmentation in general has been questioned by several investigators. For certain common types of fragmentation described here and elsewhere (Alikani et al, 1999, Hardy, 1999; Van Blerkom et al, 2001) apoptosis would seem to be precluded as an etiology of this activity because embryos were stage appropriate at transfer. For fragmented embryos that arrested development during culture, time-lapse analysis enabled TUNEL staining to be performed at precisely timed intervals after fragmentation first occurred or cytokinesis ceased (Van Blerkom et al, 2001). In this study, embryos were generally stained with TUNEL reagents within 36 hours after fragmentation was first detected and all blastomeres and fragments were reported to be TUNEL negative. Another study involved 69 embryos whose development was not monitored by time-lapse microscopy (Van Blerkom, unpublished) and TUNEL staining was performed on highly fragmented dispermic ($n=15$) and monospermic pronuclear ($n=8$; Fig. 14.1C1, 1C5) and cleavage stage embryos ($n=46$; Figs. 14.1F1, 5B1). For 64 embryos, TUNEL

FIGURE 14.4: Representative transmission electron microscopic images of fragmented human embryos at the pronuclear (A) and early cleavage stages (Figs. B-to-G). The absence of cytoplasmic organelles such as mitochondria is a typical feature of many of the extracellular fragments observed in serial section analysis of fragmented embryos in which definable nucleated (N) blastomeres were still evident. Other fragments, such as those shown in Fig. B contained mitochondria (M). Fragmented embryos that arrested cell division during early cleavage typically contained a plieomorphic population of blastomeres and fragments when examined at the fine structural level. Often, residual nucleoplast-like structures occur (N, upper right, Fig. D) which at higher magnification (Fig. E), were found to be membrane-bound structures containing scant cytoplasm and normal-appearing mitochondria (M, Fig. G). Blastomeres were occasionally vacuolated (V, Fig. F) with mitochondria and vacuoles aggregated around an intact nucleus (N, Fig. F) leaving the cortical cytoplasm devoid of organelles (asterisk, Fig. F). Cellular debris (e.g., LY, Fig. F) occurred in embryos where time-lapse analysis indicated fragment lysis. (from: Van Blerkom et al, 2001).

fluorescence was detected only in residual polar bodies (arrows, Figs. 14.1C2, 1C6, 1F2, 5B 2). In all instances, when TUNEL negative oocytes (GV, Figs. 14.1A1, 1A2; MII, Fig. 14.5A1) and fragmented embryos were exposed to DNase and restained, positive TUNEL fluorescence was observed (oocytes, arrow, Fig. 14.1A3, 5A2; embryos, Figs. 14.1F3, 5B3). Whether TUNEL fluorescence detected in highly fragmented embryos days after cell division ceases (Jurisicova et al, 1996) is the result of apoptosis or some other form of cellular degeneration will require additional investigation. However, Van Blerkom et al (2001) reported TUNEL positive staining in (a) all blastomere nuclei in cleavage stage human embryos exposed to hypotonic conditions, and (b) blastomeres that were normal and intact at cryopreservation, but which showed evident damage or lysis after thawing and rehydration. This finding suggests that TUNEL positive staining could be associated with random rather than directed (i.e., oligonucleosomal) DNA breakage. For example, the lysed thawed embryo shown in 14.1G1 exhibited intense TUNEL fluorescence (asterisks, Fig. 14.1G2) when fixed and stained 30 minutes after rehydration. In this regard, oocyte damage or lysis during ICSI (Figs. 14.5G1 and insert) is also accompanied by nonspecific DNA breakage detectable by TUNEL (Figs. 14.5G2 and insert). In these figures, oocytes were fixed for TUNEL 6 hours (Figs. 14.5G1,G2) and 10 hours (inserts) after ICSI.

Another method used to detect damaged DNA is the Comet assay, an electrophoretic procedure developed to distinguish between different forms of DNA breakage associated with cellular necrosis, autolysis, and apoptosis (Singh et al, 1988; Singh and Stephen, 1997). This assay detects DNA breaks at the single cell level and is based upon the ability of denatured, cleaved DNA to migrate from the nucleus in an agarose gel when an electric current is applied. DNA is resolved with fluorescent stains and if damaged, a 'comet' is produced where tail characteristics such as length and thickness have been associated with the cause (apoptotic, necrotic, autolytic) and nature (oligonucleosomal or random) of the breakage. In contrast, intact, supercoiled DNA remains within the confines of the nucleus and appears as a highly fluorescent 'head' with no detectable tail. We have used the Comet assay to examine the state of DNA in different types of fragmented embryos whose performance and behavior in vitro was carefully monitored either by frequent inspections or by time-lapse video-microscopy (Van Blerkom et al, 2001). Some of these fragmented embryos continued to divide while others remained arrested at the 2-to-8-cell stage. Our findings indicated TP/EH and PI-negative blastomeres (e.g., Fig. 14.5C1) did not produce evident Comets within the first 36 hours after fragmentation was initially observed (Fig. 14.5C2). However, we reported that Comets were produced by TP-positive blastomeres in highly fragmented embryos several days after cell division ceased, and the morphology of the Comets, a short, thick tail and intensely

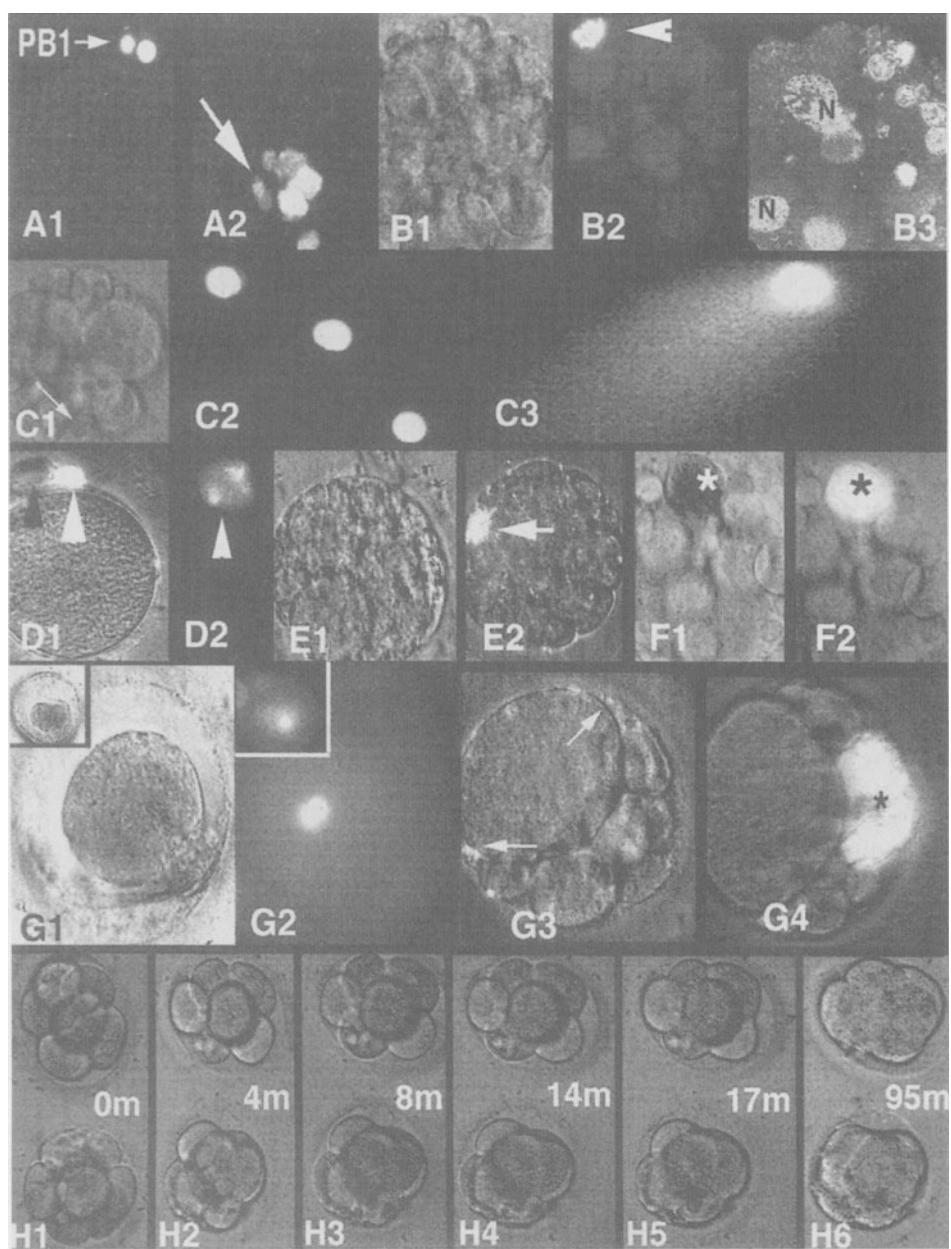
fluorescent head was consistent with a pattern of DNA damage typical of cells undergoing necrosis or autolysis rather than apoptosis (Singh et al, 1988). A very similar COMET pattern was detected in the following instances: (a) 1 hour after exposure of intact 2-to-8 cell dispermic embryos to hypotonic conditions, (b) oocytes that were damaged or which lysed during ICSI, and (c) all thawed embryos that showed significant blastomere damage, disruption or lysis. In contrast, virtually all isolated polar bodies (arrow, Fig. 14.5C1) produced Comets with long tapering tails (Fig. 14.5C3), a pattern typical of apoptotic cells in which cleavage into oligonucleosomal fragments occurs. Whether this pattern is indicative of apoptotic DNA degradation in the polar bodies as suggested for the mouse oocyte by Fujino et al (1996), or simply a manifestation of more extensive DNA degradation in these unique structures remains to be determined.

Liu et al (2000) and Liu and Keefe (2000) reported that a 15-minute exposure of pronuclear and 2-cell stage mouse embryos to hydrogen peroxide at levels that induce 'mild' oxidative stress was associated with an immediate arrested cell division. However, after several days of culture, indications of apoptosis were observed which included generalized (i.e., non-mitochondrial) cytoplasmic cytochrome c immunostaining, caspase-3 immunofluorescence, TUNEL staining, loss of mitochondrial hyperpolarization, and changes in mitochondrial fine structure. A finding that may be of particular relevance for the human was that biochemical and fine structural changes involving mitochondria were first detected 72 hours after treatment, suggesting that the occurrence of mild oxidative stress during the earliest stages of mouse development may be induce PCD and apoptosis several days later. The primary effect of induced oxidative stress was shown to occur at the cytoplasmic level and was most likely associated with disruption of mitochondrial function leading to a delayed release cytochrome c from these organelles and activation of cytoplasmic procaspase-3 (Marchetti et al, 1996; Matsuyama et al, 2000). Eventual cell death for pronuclear and 2-cell mouse embryos was accompanied by cell shrinkage, an activity suggested by these investigators to be a characteristic of apoptosis in mouse embryos. If a similar process is involved in human embryo fragmentation, potential extrinsic sources of oxidative stress and the origin of toxic levels of intrinsically generated reactive oxygen species (ROS) need to be identified. Within cohorts, fragmentation primarily involves only some embryos or blastomeres. In this sense, it seems unlikely that developmentally lethal levels of oxidative stress-inducing agents occur in culture media since the effects might be expected to be universal, as in mouse embryos exposed to nonphysiological levels of hydrogen peroxide (Liu and Keefe, 2000). In addition, current formulations of human embryo culture media contain a variety of

antioxidants, including maternal serum in some formulations, which have been suggested to minimize the potential for iatrogenic oxidative stress.

Interpretations of cell death from treated mouse embryos are difficult to reconcile with spontaneous fragmentation activities and phenotypes observed in cultured human embryos. In the context of results obtained by time-lapse analysis, fragmentation may be accompanied by a slight reduction in cell volume, but the ability of the affected cell to divide is often not compromised.

FIGURE 14.5. TUNEL staining and the Comet assay were used to detect DNA breakage in oocytes and fragmented human embryos. Figure A1 shows TUNEL fluorescence in both compartments of a first polar body that had divided in a normal uninseminated MII oocyte. After DNase treatment, cytoplasmic chromosomal TUNEL fluorescence was evident (arrow, Fig. A2). Figures B1-B3 are light (Fig. B1) and TUNEL fluorescent images of a fragmented 8-cell embryo that at the 4-cell stage, contained several multinucleated blastomeres. TUNEL fluorescence was associated with a single peripheral structure (arrow, Fig. B2) which appears to be the second polar body. After DNase treatment, nuclear TUNEL fluorescence was intense and present in all blastomeres (Fig. B3). Figures C1-C3 are representative light (Figs. C1) and fluorescent images (Figs. C2-C3) of a fragmented early cleavage embryo examined by the Comet assay. Blastomere nuclei appeared as intensely fluorescent spheres with no evident tail (Fig. C2). In contrast, second polar bodies examined after removal from the embryo showed a classic ‘Comet’ pattern (Fig. C3) indicative of extensive DNA fragmentation. Figure D1 shows annexin V (AV) staining of one compartment of a divided first polar body. As shown in figure 5D2, this compartment was also positive after staining with propidium iodide (PI) indicating loss of plasma membrane integrity. For highly fragmented cleavage stage embryos, AV staining was detected in a few fragments (arrows, Figs. 5E1,E2, asterisk, Fig. 5F2) that were also PI or trypan blue positive (Fig. 5F1). Uninseminated or unfertilized oocytes occasionally segmented after several days of culture. In most cases, only small areas of AV fluorescence occurred (day 5 of culture, arrows Fig. 4G3), while in others, the entire cytoplasm of one or two large segments was AV positive (Fig. 5G4). TUNEL fluorescence was detected in oocytes that lysed after intracytoplasmic sperm injection (ICSI). In figures 5G1 and insert, lysis occurred 30 minutes and 2 hours after ICSI, respectively, and both showed intense chromosomal TUNEL staining indicative of nonspecific DNA breakage. Figures 5H1-H6 show the affects of the apoptosis-inducing kinase inhibitor staurosporine (STR) on normal 8-cell embryos during the first 95 minutes of treatment (times indicated in middle of each figure). Exposure to STR was associated with a rapid compaction-like activity and during additional culture, cell division continued in compacted embryos.



Where very significant cytoplasmic loss has occurred and cell division ceases, the residual cell(s) can remain intact for days in culture (Van Blerkom et al, 2001) with no evident mitochondrial swelling or fine structural alterations (e.g., Fig. 14.4G) similar to those described for early mouse embryos exposed to hydrogen peroxide. We have also reported that loss of a human blastomere can occur by abrupt swelling and lysis rather than shrinkage (Antczak and Van Blerkom, 1999). While the origin(s) of developmentally lethal patterns of fragmentation in human embryos is unknown, the possibility that unique physiological conditions exist at the embryo or blastomere levels which convey a predisposition to fragmentation by apoptosis or other means is an intriguing one, especially if associated with specific culture conditions or a differential capacity to buffer ROS.

CAN APOPTOSIS BE EXPERIMENTALLY INDUCED IN HUMAN OOCYTES AND EMBRYOS?

The detection of pro-and anti-apoptotic proteins in human oocytes and early embryos has suggested that the capacity to activate PCD pathways resulting in apoptosis exists at the earliest stages of development (Liu et al, 2000; Jurisicova et al, 2003). We have examined the response of normal human oocytes and cleavage stage embryos to various agents known to induce PCD and apoptosis in cultured cells and here we report findings with one widely used agent, staurosporine (STR). STR is a bacterial alkaloid inhibitor of protein kinases involved in intracellular signaling pathways (Tamaoki and Nakano, 1990), and has been reported to be a nonphysiological inducer of PCD and apoptosis in a wide variety of cell lines and dissociated primary cell cultures (see, Weil et al, 1996; Yoshida et al, 1997). STR induces mitochondria permeability transition and release of cytochrome c and other proapoptotic proteins from the intramembranous space of this organelle (Tafani et al, 2001), which in turn activate cytoplasmic elements of the PCD pathway such as caspases (Hortelano et al, 1997). STR has also been suggested to have similar effects on MII-stage mouse oocytes and preimplantation stage embryos (Weil, et al, 1996; Warner et al, 1998; Exley et al, 1999).

Weil et al (1996) cultured unfertilized mouse eggs and isolated blastomeres from 2-or 4-cell embryos for up to 24 hours in 1 μ M STR, either in the presence or absence of the protein synthesis inhibitor cyclohexamide. These authors reported negative TUNEL staining of chromosomal DNA in oocytes, nuclear DNA in intact 4-cell embryos and mechanically disaggregated blastomeres, and no uptake of EH. However, when exposed for up to 60 hours to STR at a concentration of 10 μ M, most oocytes and embryos died. Blastomeres from these embryos were EH positive and the

corresponding nuclei were described as pyknotic and TUNEL positive. Weil et al (1996) concluded that unlike other cell types that undergo PCD when treated with STR at similar concentrations and for similar durations, mouse oocytes and early blastomeres are unusually resistant to the lethal effects of this drug. However, they interpreted cell death and TUNEL positive staining after extended exposure at high concentration as indicative of an inherent capacity of oocytes and blastomeres to activate PCD and undergo apoptosis. The apparent activation of PCD in blastomeres where translation was inhibited by cyclohexamide suggested that proteins involved in a putative PCD cascade were pre-existing. In cultured cell lines, the effects of STR follow a more typical pattern of apoptosis induction with cytoplasmic and nuclear degeneration followed by disintegration into apoptotic bodies occurring over hours rather than days. For example, Godard et al (1999) followed the time course of STR-induced apoptosis in Chinese hamster ovary cells and reported AV staining after a 1-hour exposure, DNA Comets after 3 hours, while positive TUNEL staining was not detected until 6 hours.

We examined the effects of STR on human oocytes and early embryos under the following circumstances: (a) normal appearing MII oocytes that matured in vitro from the GV stage ($n=17$), (b) newly retrieved uninseminated MII oocytes ($n=9$), (c) unfertilized oocytes (24 hours post insemination, $n=39$), and (d) normal appearing 8-cell embryos (day 3: monospermic, $n=4$; dispermic, $n=13$). Oocytes and embryos were cultured continuously in the presence of 10 μ M STR. During 48 hours of culture, oocytes remained intact and were AV, Comet, and TUNEL negative. For embryos, however, a rapid, compaction-like process occurred within minutes after the addition of STR, as shown in time-lapse sequence in figures 14.5H1-H6. For 62% of the prematurely compacted embryos (18/29), culture in the presence of STR for 24 hours was accompanied by cell division to 12-to-16 cell stage (determined by nuclear DNA fluorescence), and active motility of blastomere plasma membranes was observed by time lapse imaging. For both progressive and nonprogressive embryos, no cell-specific AV staining, TUNEL fluorescence, or COMET tails were detected after 36 hours of treatment. After approximately 40 hours of culture, membrane motility ceased and by 44 hours, PI or TP incorporation was detected in some blastomeres and in all cells by 60 hours. All PI/TP positive blastomeres were TUNEL and AV positive. Sixty hour old-embryos ($n=6$) produced short, thick Comet tails that have been found to be characteristic of random DNA breakage (Singh and Stephen, 1997). The rapid and premature compaction-like activity of STR-treated embryos may be related to the known effects of this drug on a broad spectrum of cellular activities including influences on protein kinases involved in intracellular signaling pathways (Yoshida et al, 1997).

PCD and apoptosis have been proposed as a means by which normal cells can be eliminated from the embryo at the blastocyst stage (Hardy, 1999), and therefore may be a normal feature of development at late pre- and early post-implantation stages (Brison and Schultz, 1997). In this sense, the capacity to activate PCD in response to intrinsic cell death signals or to extrinsic influences such as STR may be stage-specific or possibly, require new gene expression associated with full activation of the embryonic genome. While some components of the apoptotic pathway have been detected in oocytes and early embryo, perhaps this pathway is functionally incomplete at the very early stages of human development. This possibility could explain why agents such as STR appear to induce a rapid apoptosis-like response at the blastocyst stage but not at the early cleavage stages (Weil et al, 1996; Brinson and Schultz, 1997; Warner et al, 1998; Exley et al, 1999; Hardy, 1999). For the human, we are currently investigating whether stage-specific PCD and apoptotic capacities exist and whether in order to respond to known inducers of apoptosis, a functional PCD pathway is associated with major embryonic genomic activation. We are also investigating whether higher concentrations of STR may be required to induce an apoptotic-like process in MII oocytes and early human embryos.

None of the above results preclude the possibility that under certain instances, fragmentation during the early cleavage stages of human development is a manifestation of an apoptotic process. However, the general assumption in clinical IVF that fragmentation is indicative of apoptosis needs to be placed in context with both the extent of fragmentation, loss of blastomeres, the capacity for cell division to continue, and with respect to cell numbers, the generation of stage-appropriate embryos. While the molecular detection of proteins involved in PCD and apoptosis demonstrates that components of cell death pathways exist in human oocytes and early human embryos, whether they are active in apoptosis at these stages needs to be determined unambiguously. In this regard, a recent study by Martinez et al (2002) is illustrative of an association between expression of an apoptotic protein and fragmentation in human cleavage stage embryos that seems to be more apparent than real. These investigators used a universal fluorochrome caspase inhibitor (FITC-VAD-FMK) to visualize active caspases in blastomeres and fragments of 2-to-12 cell human embryos. In these studies, cytoplasmic fluorescence was indicative of the conversion of procaspses (i.e., caspase-3) to an active form resulting in the liberation of a fluorescent tag (FITC), an activity which in somatic cells is a marker of apoptosis. The study examined several hundred blastomeres in good and poor morphology (i.e., fragmented) embryos and the results demonstrated rare caspase positive blastomeres in only very poor morphology embryos and embryos containing multinucleated blastomeres. For highly fragmented embryos, less than half of

the fragments associated with a caspase-negative blastomere(s) were fluorescent. In studies described above, we detected TUNEL fluorescence in and the generation of Comets from the second polar body, and in the Martinez et al (2002) report, active caspase activity occurred in these structures as well. The fact that two markers of DNA fragmentation and one biochemical marker of apoptosis in living cells occur in a structure which remains intact throughout the preimplantation stages, to the extent that it can serve as a geographical marker related to the establishment of axes of polarity at the blastocyst (see Scott, this volume), raises an interesting question concerning their association with and specificity for a genetically-driven cell death pathway in the early human embryo. More importantly, Martinez et al (2002) reported that caspase positive fragments were found in association with healthy blastomeres, a finding derived from the fact that when cells were removed from an embryo and associated caspase positive and negative fragments eliminated by micromanipulation, the individual blastomeres divided in culture. In these instances, new fragments formed, some of which were caspase positive, while the underlying blastomeres were negative. These investigators concluded that active caspases might be involved in developmental or formative processes of early human embryogenesis unrelated to cell death. If the specificity of this probe is confirmed, active caspases could have a role in intracellular remodeling, perhaps by initiating a stage-or cell-cycle-specific relaxation of the cytoskeletal system, which is one target of these potent cysteine proteases. The origin of the differential caspase activity detected in some fragments but not in adjacent structures needs to be determined. Martinez et al (2002) suggested that differences in mitochondrial content between fragments might be related to pathological effects on mitochondria that are both fragment-specific and associated with cytochrome c release, which would convert pro-caspases to an active form. Continued investigation is warranted and may provide important insights into the differential origin, behavior and fate of fragments observed by time-lapse microscopy and with respect to mitochondria as discussed below.

It seems that where fragmentation is so severe and complete as to be lethal to the early human embryo, apoptosis is the most likely cause of demise. The most pressing questions in this regard are the frequency with which complete fragmentation occurs in different culture media and conditions, and in particular, why only specific embryos within and between cohorts are affected. What will be especially relevant for experimental analysis is whether intrinsic threshold differences in pro- and antiapoptotic proteins exist between normal appearing embryos and what unique internal or external factors ultimately influence the molecular decisions that tips the balance between survival and death. The identification of specific molecular, cellular or genetic defects that result in the activation of apoptosis during the early stages of

human embryogenesis would go a long way in providing both patient-specific and fundamental biological insights into why certain embryos self-destruct shortly after fertilization. In clinical IVF, the central issue is one of whether this form of wastage is intrinsically or extrinsically regulated, or both.

POTENTIAL NONAPOPTOTIC CAUSES OF FRAGMENTATION

While PCD and apoptosis may be responsible for some forms of severe fragmentation commonly observed in cultured human embryos, these cell death mechanisms cannot explain the occurrence of developmentally competent, stage-appropriate human embryos that show fragmentation patterns of varying severity. Because cytokinesis is a dynamic process involving relatively rapid changes at the cytoplasmic and plasma membrane levels, some fragments may simply represent remnants of the plasma membrane and corresponding cytoplasm that become separated from blastomeres as a normal consequence of the cleavage process. Possible causes of severe fragmentation associated with significant reductions in blastomere volume are more difficult to explain when such blastomeres retain an intact nuclear membrane, show no indication of cytoplasmic or organelle degradation, and are AV, TUNEL, or Comet negative.

ARE MITOCHONDRIAL METABOLISM AND FRAGMENTATION RELATED?

One of the more interesting characteristics of fragmentation in cultured human embryos is that it largely confined to the early cleavage stages. This consistent observation led us to ask whether different forms of fragmentation could have a metabolic basis associated with blastomere-specific differences in mitochondrial content or the capacity to generate ATP by respiration (oxidative phosphorylation, OXPHOS) which is a primary source of energy for the cleaving human embryo (Cummins, 2002). Defects in mitochondrial function that could lead to reduced ATP production or to the generation of high levels of ROS could have deleterious, if not lethal effects on cell structure and function, including the ability of the cytoplasm to undergo spatial remodeling and maintain plasma membrane function and integrity. For example, neuromuscular degenerative diseases that develop in individuals with certain maternally-inherited mitochondrial DNA (mtDNA) disorders (OXPHOS diseases) are largely associated with reduced metabolic capacity in cell types that have high demands for ATP (Naviaux and McGowan, 2000; Trounce, 2000). For affected individuals, the consequences of inherited OXPHOS disorders can range from debilitating-to- lethal depending upon the

extent (mutant load) of heteroplasmy (the occurrence within a cell of different mitochondrial genotypes) involving mutant mitochondria with respiratory defects. For individuals with normal mtDNA, age-related deterioration in nerve, muscle, and liver cell function has long been associated with a progressive accumulation of mutant mitochondria, and at advanced age, cellular dysfunction or reduced activity can be largely attributed to the effects of diminished metabolic capacity.

For human sperm, multiple deletions in mtDNA have been correlated with reduced motility and fertilization potential (Lestienne et al, 1997; Kao et al, 1998). The potential relationship between human mitochondrial function and fertility has focused on the identification and characterization of mutations in mtDNA as possible causes of reduced competence for oocytes and early embryos in general (Chen et al, 1995; Brenner et al, 1998; Barrit et al, 1999, 2000), and for women of advanced reproductive age in particular (Keefe et al, 1995). The rationale for this approach stems from the fact that mitochondria are maternally inherited and the complement of approximately 150,000 organelles thought to occur in the mature human oocyte is derived from a very small number of progenitors, perhaps <10 (Jansen and DeBoer, 1998), in the primordial germ cell. Consequently, heteroplasmy may occur if the replication of the mitochondrial population during oogenesis includes preferential expansion of progenitor(s) with altered mtDNA resulting in an oocyte with a high mutant load. Because mitochondrial replication in the embryo begins after implantation, the potential adverse consequences of heteroplasmy on competence could result from reduced metabolic efficiency if the mutation(s) effects respiratory chain enzymes, and cytoplasmic glycolytic metabolism is unable to compensate for reduced mitochondrial ATP output. As noted by Christodoulou (2000), the random segregation of inherited mitochondria among cells during the preimplantation stages produces a potential wide range of mtDNA genotypes from virtual homoplasmy for the wild-type mtDNA to virtual homoplasmy for the mutant DNA, and at a certain mutant load, a threshold is reached beyond which cellular dysfunction will become evident. While it is tempting to conclude that human oocyte and embryo competence may have a mitochondrial genetic basis (see Brenner, this volume), such an assumption needs to consider the occurrence of infants born with mitochondrial-related OXPHOS diseases where mtDNA mutations are known to effect respiratory chain enzyme function and metabolic capacity (Chinnery et al, 1999; Leo and Schapira, 2000). This implies that fully efficient respiratory chain function is not critical during human oogenesis or early embryogenesis, and developmental incompetence for the oocyte or early embryo may occur only when mutant loads are exceedingly high (Shoubridge, 2000).

The notion that an increased mtDNA mutant load in the oocytes of women of advanced reproductive age may reduce metabolic capacity or be associated

with toxic levels of ROS are intriguing possibilities, especially if the frequency of fragmentation was also age-related in the early embryo. It is unknown whether instances of early human embryo demise, including those accompanied by fragmentation, could result from a heteroplasmy-associated energy deficiency (Jansen 2000) and to date, the magnitude of detected heteroplasmy in some infertile women is well below levels that would be considered developmentally lethal. In addition, there is no compelling evidence from clinical IVF that the frequency or patterns of fragmentation in the early embryos of older women are any different from those in younger women (Alikani et al, 1999). However, the possibility that mitochondrial metabolic activity may be an important determinant of competence was suggested by studies of the net ATP content of randomly selected uninseminated MII human oocytes in cohorts where sibling oocytes were inseminated and transferred on day 2 or 3 (Van Blerkom et al, 1995). Although this study involved a relatively small number of patients, the findings indicated the following: (a) the net ATP content of normal appearing MII oocytes could differ by nearly an order of magnitude, (b) when the majority of uninseminated oocytes within a cohort were at the low end of the range, the ongoing pregnancy for normal appearing embryos from sibling oocytes was significantly lower than observed with (c) morphologically equivalent embryos from cohorts where superfluous oocytes had ATP contents at higher levels. While this finding was suggestive of a metabolic association with competence, it could not explain the basis for such differences between oocytes in the same and different cohorts. One possibility is that the size of the mitochondrial complement differs significantly between MII oocytes that appear equivalent at the light microscopic level.

Several years ago, we quantified the number of copies/oocyte of the mitochondrial gene ATPase 6 (a subunit of ATPsynthase) in 62 normal appearing, donated MII oocytes with mtDNA extracted within 2 hours of ovum retrieval for ZIFT and GIFT procedures. The findings indicated that ATPase 6-copy number was oocyte-specific and was estimated between 40,000 to nearly 700,000 copies/oocyte, with an average copy number of approximately 205,000. To date, this work has remained unpublished because until recently, we thought that it unlikely that mtDNA copy number between MII oocytes could differ by well over an order of magnitude and we considered these values to be unreliable, possibly an artifact associated with mtDNA preparation or the conditions of the polymerase chain reaction. However, other quantitative analyses of mtDNA have shown very different mtDNA copy numbers/oocyte, with reported averages of 138,000 (Chen et al, 1995), 193,000 (Reynier et al, 2001) and 314,000 (Steuerwald et al, 2000). Taken together, the findings suggest copy numbers between approximately 20,000 to over 600,000 can occur in normal appearing oocytes from the same

and different cohorts, with 1-to-10% of mtDNA located in the first polar body (Steuerwald et al, 2000). With respect to competence, Reynier et al (2001) reported that fertilization failures occurred in most MII oocytes with mtDNA copy numbers less than 50,000, suggesting that some proportion of IVF failures could be due to inadequate oocyte "maturation" that resulted in numerical defects in mitochondrial biogenesis. While the relationship between fertilizability and mtDNA copy number needs to be confirmed, the findings could indicate that for oocytes with normal mtDNA, the size of the mitochondrial complement, rather than the possible occurrence of low-level heteroplasmy, may be a critical determinant of competence.

The prevailing notion that each oocyte mitochondrion contains a single mtDNA genome (Shoubridge, 2000, Cummins, 2002) implies that the absolute number of mitochondria in oocytes used in clinical IVF may differ by well over an order of magnitude. While human oocyte mitochondria are readily identifiable in electron microscopic images, quantitative assessments of the cytoplasmic complement cannot be made from a single thin section. Muller-Hocker et al (1996) used a morphometric approach to determine mitochondrial volume (mitochondrial profile area, μm^2) and numerical density (numbers of mitochondria/ μm^2 and per μm^3) in 41 oocytes obtained from 36 women aged 27-39 years undergoing ovarian hyperstimulation for GIFT. The findings indicated a progressive age-related increase in both profile area and apparent cytoplasmic density. The authors proposed that an age-related increase in mitochondrial density could be an adaptive mechanism during oocyte growth to compensate for reduced respiratory efficiency/mitochondrion by increasing the number of mitochondria, albeit with diminished metabolic capacity/unit. A trend towards an age related increase in mtDNA copy number was also reported by Steuerwald et al (2000). However, Muller-Hocker et al (1996) found no evidence for an age-related increase in the frequency of point mutations in mtDNA or functional defects of the respiratory chain complexes III and IV, suggesting that genetic or potential metabolic defects are not prevalent in mature oocytes. Whether the accuracy of the morphometric sampling method used by these investigators could be effected by oocyte-specific differences in the spatial distribution of mitochondria remains to be determined and therefore, other morphometric and analytical methods may be required to establish whether the absolute number of mitochondria in human oocytes can differ by ten or twenty-fold, and whether some proportion of mitochondria contain multiple genomes. The findings that mtDNA copy number and apparent numerical cytoplasmic density can differ significantly between seemingly equivalent MII oocytes, and that these differences may be related to developmental competence, suggests that the relative size of the mitochondrial complement may be a critical determinant of the oocyte and early embryo viability.

If the capacity to generate ATP is related to the competence, why do human oocytes with relatively low mtDNA copy numbers (Reynier et al, 2001) and comparatively low cytoplasmic ATP contents (Van Blerkom et al, 1995) complete meiotic maturation and appear normal at the light microscopic level, especially if cytoplasmic remodeling and nuclear progression associated with normal preovulatory oocyte maturation are energy-requiring processes? One possibility is that different metabolic thresholds exist for oocyte maturation and early embryonic development, and that the relative ATP contribution from mitochondrial respiration and cytoplasmic glycolysis is stage-related. The mitochondrial contribution would be expected to reflect the number of normally functioning organelles present both in the oocyte at fertilization and in each cell of the developing embryo. If it is assumed that in the absence of replication, the mitochondrial complement is approximately halved with each cell division, and that segregation between daughter cells is largely equivalent, the potential consequence of reduced mitochondrial numbers may become evident as development progresses through cleavage, especially if normal cytoplasmic glycolytic pathways are unable to compensate for a diminished mitochondrial contribution in the human. According to this notion, the size of the mitochondrial complement, which may or may not be reflective of mtDNA copy number, may be sufficient to support nuclear and cytoplasmic processes associated with meiotic maturation, even at the low end of the range reported for MII human oocytes (Van Blerkom et al, 1995), but insufficient to support cellular energy requirements for biosynthetic and morphodynamic activities during the preimplantation stages. As described below, we suggest that for specific blastomeres, certain morphodynamic activities and adverse developmental consequences could be associated with disproportionate mitochondrial inheritance during early cleavage.

COULD DIFFERENT FRAGMENTATION PHENOTYPES BE RELATED TO BLASTOMERE-SPECIFIC METABOLIC CAPACITY?

For the human embryo, putative deficiencies in mitochondrial numbers could have a spectrum of developmental consequences including arrested cytokinesis, fragmentation, or blastomere lysis, if ATP levels fall below threshold levels required to maintain plasma membrane ion pumps and normal transport activities (Van Blerkom et al, 2001). When we examined the net ATP contents of intact dispermic and fragmented monospermic cleavage stage human embryos from the same cohorts, levels in the fragmented embryos were generally comparable to those measured in intact siblings, although for some fragmented embryos, significantly lower ATP contents

were detected (Van Blerkom et al, 1995). This finding suggests that measurement of the net ATP content of whole embryos may not be of sufficient sensitivity to make clear associations with fragmentation. However, we have recently asked whether activities in individual blastomeres, including fragmentation, could be related to the relative size of the mitochondrial complement and the net ATP content of the cytoplasm. For these studies, metabolically active mitochondria were labeled with organelle-specific fluorescent probes and their spatial distribution was examined in (a) intact embryos between the pronuclear and cleavage stages (Van Blerkom et al, 2000), and (b) in blastomeres and fragments (Van Blerkom et al, 2001).

Analyses of normal pronuclear embryos demonstrated varying degrees of asymmetry in mitochondrial distribution within the cytoplasm in general and peri-pronuclear nuclear region in particular. In certain instances, the asymmetry was so pronounced as to leave large portions of the cytoplasm relatively devoid of mitochondrial fluorescence. As the progress of development was monitored during culture, mitochondrial inheritance between daughter blastomeres was often disproportionate and could be related to how the plane of the first cleavage division bisected the 1-cell embryo. In other instances, diminished inheritance within one blastomere at the 2-cell stage was observed as reduced mitochondrial fluorescence in progeny blastomeres between 4-and-6-cell stages. Where the spatial distribution of mitochondria at the pronuclear stage was classified as symmetrical, the intensity of mitochondrial fluorescence between blastomeres through early cleavage was largely equivalent. Biochemical analysis of single blastomeres showed a direct correlation between the intensity of mitochondrial fluorescence and net ATP content for embryos in which the spatial distribution of mitochondria at the pronuclear stage was characterized as symmetrical or asymmetrical.

The fate of blastomeres with disproportionate segregation from the 1-cell stage that resulted in reduced mitochondrial inheritance was of particular clinical interest because it could explain certain instances of blastomere arrested or seemingly delayed embryonic development observed with cultured human embryos (Van Blerkom et al, 2000). Our findings showed that blastomeres with poor mitochondrial fluorescence remained intact and undivided, but during culture, often underwent cytoplasmic changes that led to an abrupt swelling and subsequent lysis. Prior to lysis, blastomeres were either AV- or TUNEL- negative. This pattern of demise seems to be distinctly different from both delayed cell death described by Liu et al (2000) and Liu and Keefe (20001) for hydrogen peroxide-treated mouse embryos, and from classical descriptions of experimentally-induced somatic cell apoptosis. Serial section TEM analysis of blastomeres that exhibited poor mitochondrial fluorescence showed a comparatively diminished or significantly reduced

mitochondrial complement (Van Blerkom, unpublished). We have detected no evident pathological disorders for mitochondria in these cells (similar to mitochondrial profiles shown in Fig. 14.4G) such as described by Liu et al (2000) for ‘stressed’ and arrested pronuclear and 2-cell mouse embryos. For the mouse, mitochondrial alterations were suggested to be related to cytochrome c release, caspase-3 activation, and apoptosis (Matsuyama et al, 2000). Morphometric analysis will be required to determine the extent to which mitochondria may be over-represented in some human blastomeres at the 2- to- 8-cell-stage. Likewise, whether elevated levels of ROS that could adversely effect cytoplasmic structure and organization and lead to fragmentation occur in these blastomeres will require biochemical analysis. However, because high levels of endogenously generated ROS can be an inducer of apoptosis in somatic cells, for the human embryo, confirmation of a clear correspondence between cell-specific ROS levels, mitochondrial content, metabolic activity, and the occurrence of molecular markers of apoptosis (e.g., cytochrome c release, caspase activation, AV staining, long and tapered Comet tails) would be strong evidence in support of PCD activation and an apoptotic basis of fragmentation in these instances.

The relationship between cytoplasmic ATP generation/levels and the mode of cell death observed in some cultured cell lines may provide important insights as to whether a similar relationship exists in human embryos (Richter et al, 1996; Leist et al, 1997; Lelli et al, 1998). Leist et al (1997) demonstrated that cytoplasmic ATP concentration was a “switch” in the decision between apoptosis and necrosis in human T cells (Jurkat cells) exposed to two well known apoptotic triggers, STR and α CD95. These investigators determined the mode of cell death in treated cells under conditions of intracellular ATP depletion and repletion by blocking mitochondrial respiration with oligomycin, and by modulating glycolytic and mitochondrial ATP generation by varying glucose and pyruvate concentrations in culture media, respectively. Necrotic cell death occurred under conditions of reduced ATP concentration. In contrast, when cytoplasmic ATP levels were increased experimentally, cell death occurred by apoptosis. In the case of necrotic cell death, intact cells contained nuclei of normal size with no evidence of apoptotic chromatin condensation, and no detectable cleavage of nuclear lamin B, whose occurrence in apoptotic cells involves activation of specific caspases and is required for nuclear collapse (Lazebnik et al, 1995). Molecular analysis showed no AV staining of the plasma membrane and the absence of DNA fragmentation or cleavage into oligonucleosomal-sized fragments, the hallmark of apoptotic cell death. With continued metabolic inhibition, AV staining was detected during cell swelling which preceded lysis. In contrast, apoptosis occurred under conditions of normal or replenished cytoplasmic ATP and was confirmed by AV staining,

chromosomal condensation on the nuclear membrane, lamin B degradation, and endonuclease-mediated cleavage of DNA fragments into oligonucleosomal fragments.

Several other findings and conclusions reported by Leist et al (1995) may be relevant in regard to apparent degenerative processes that occur in cultured embryos. When the mode of cell death was correlated with specific ATP concentrations, ATP depletion >50% changed demise from apoptosis to necrosis. While higher concentrations promoted the continuation of apoptosis, concentrations <70% of normal invariably resulted in necrosis. By modulating cytoplasmic ATP levels and the time of treatment with apoptosis inducers, demise could be shifted between apoptosis and necrosis and between necrosis and apoptosis. Between 30% and 50% depletion, intermediate forms of necrotic cell death were observed, such as the occurrence of partially condensed chromatin. Under conditions of mitochondrial metabolic inhibition, the level of cytoplasmic ATP supplied by glycolysis was sufficient to support the ordered execution of apoptotic cell death. One of the more salient conclusions of this study can be paraphrased as follows: 'apoptosis and necrosis are two extremes of a continuum of possible types of cell demise that may be decided by the availability of ATP, which may explain the frequent coexistence of both types of cell death in pathological situations where individual cell death within a tissue could be decided on the basis of the energy supply.'

For the human embryo, the work of Leist et al (1995) could reconcile differences in the interpretation of cell behavior and death in preimplantation stage human embryos described earlier in this chapter. During the cleavage stages, where mitochondria are considered a significant source of energy, ATP concentrations in specific blastomeres may occur below threshold levels required to maintain normal cellular activities and functions. While findings from individuals affected with known maternally derived mitochondrial genetic defects indicate that the presence of a fully functional respiratory chain is not lethal for the oocyte or preimplantation stage embryo, the actual concentrations of ATP present in affected oocytes and embryos are unknown, but may be presumed to be above critical threshold levels. In contrast, if mtDNA copy number in the oocyte is an accurate indicator of the size of the complement, subthreshold levels may occur in essentially homoplasmic embryos with inherited numerical deficiencies, or where disproportionate segregation has occurred during cleavage.

If the above findings from somatic cell lines are applicable to activities in early human embryos, then perhaps some of the fragmentation patterns observed may represent different pathways of cell demise in a continuum between necrosis at one end and apoptosis at the other. Evidence from embryo performance in vitro and outcome in vivo indicates that many common forms

of fragmentation have no adverse or long-term effects on development, including those, which appear to be of pseudo-fragment type. In this regard, we have suggested that some types of fragmentation resemble activities associated with oncosis (Van Blerkom et al 2001), which could be an intermediate in the continuum between necrosis and apoptosis. Majno and Joris (1995) have described in detail the cellular and molecular characteristics of oncosis and in particular, how it differs from apoptosis. Oncosis is characterized by an initial elaboration of largely organelle-free blebs of the plasma membrane, and subsequently by increased plasma membrane permeability, cellular swelling and lysis. AV staining and nonspecific DNA degeneration detectable by TUNEL occur at the terminal stages of oncosis. Blebbing of the plasma membrane is a direct result of diminished cytoplasmic ATP concentration that leads to reduced efficiency and ultimate failure of plasma membrane-associated ionic pumps, with cell lysis the final result.

The formation and behavior of blebs produced during the early stages of oncosis are of particular interest with respect to certain types of fragment and pseudofragments formation in early human embryos. In somatic cells undergoing oncosis, these structures have been reported to be largely organelle-free and arise from the plasma membrane as fluid-filled protrusions containing some of the cortical cytoplasm. The protrusions can become detached from cell surface or retain continuity with the affected cell. Coincident with plasma membrane activity, mitochondria become internally located. If cytoplasmic ATP levels fall below a critical threshold, the protrusions swell and lysis, leaving behind a largely intact nucleus, a residual cytoplasmic matrix, remnants of the plasma membrane, and intact blebs that had previously detached from the cell membrane. Oncosis has been described for certain types of cells experiencing oxygen deprivation (ischemic conditions). However, if appropriate conditions are restored within a time sufficient to adequately replenish cytoplasmic ATP reserves by normal mitochondrial respiration, recovery is associated with the resorption of blebs and return of normal cell function. For some cells, oncosis may be an adaptive mechanism of cytoplasmic remodeling and reduction that permits short-term survival under adverse conditions. However, if these conditions persist for prolonged periods, the result is autolytic or necrotic-like cell death. It is also important to note that in some cells ischemia can also induce apoptosis (Martinou et al, 1994) so that the initial response pathway to adverse conditions may be cell type specific.

As shown here and elsewhere (Van Blerkom et al 2001), a paucity or virtual absence of mitochondria is one fine structural characteristic common to many detached fragments in general, and fragments in columns in particular, especially where cytoplasmic continuity with the associated blastomere(s) seems to persist. This finding was confirmed by staining with

mitochondria-specific fluorescent probes (Van Blerkom et al, 2001). These features are similar to descriptions of the initial stages of oncosis discussed by Majno and Joris (1995). We have speculated that certain benign forms of fragmentation at both pronuclear and early cleavage stages may result from localized blebbing of the plasma and subjacent cytoplasm. The finding that a significant proportion of the mitochondrial complement is centrally located at the pronuclear at early cleavage stages (Van Blerkom et al, 2000,2001) may explain the comparative absence of these organelles in both columns of putative pseudofragments and detached fragments observed at the fine structural and fluorescent microscopic levels. The absence of mitochondria in detached fragments is likely to be associated with diminished ATP reserves which cannot be restored by glycolysis, and over time may lead to swelling and autolysis similar to what is observed under conditions of prolonged respiratory distress leading to oncosis.

If certain forms of fragmentation in human embryos are energy-related, how could such ATP deficiencies occur and why are only some embryos or blastomeres affected? Asymmetrical mitochondrial distributions at the pronuclear and early cleavage stages may leave portions of the cortical cytoplasm at subthreshold levels of ATP such that focal blebbing of the plasma membrane may occur as an adaptive oncotic-like response to an ATP deficit. The severity of asymmetrical distributions and effects on competence may also be related to the size of the mitochondrial complement (or mtDNA copy number) inherited from the oocyte. According to this notion, which is currently under study, embryos derived from oocytes with subnormal mitochondrial inheritance, or where mitochondrial distribution between blastomeres is severely disproportionate, may be more prone to certain forms of oncotic-like fragmentation. The apparent resorption of fragments could occur if threshold levels of ATP are restored as a result of stage- and cell cycle specific spatial remodeling of the cytoplasm during the pronuclear and early cleavage stages, processes which are normally associated with demonstrable intracytoplasmic motility and circulation (Edwards and Beard, 1997; Payne et al, 1997). In the absence of compensatory glycolytic ATP generation, severely reduced mitochondrial inheritance, if accompanied by diminished respiratory activity, could lead to extensive blebbing of the plasma membrane, which for the affected blastomere(s), could generate detached fragments with varying mitochondrial contents. The level of this activity, the cell(s) affected, and the stage(s) of cleavage when it is observed could be subject to blastomere-specific mitochondrial content as related to threshold levels of ATP. At the blastomere level, reduced ATP reserves may first effect the capacity to divide and subsequently reach levels insufficient to maintain cellular functions and plasma membrane integrity. In contrast to apoptosis, where nuclear and cytoplasmic deterioration leads to apoptotic body

formation, an oncotic-like process could explain the occurrence of extracellular fragments that often surround an intact blastomere that, while significantly reduced in size, remains intact with no evident nuclear membrane or DNA deterioration. Whether detached fragments persist or lyse may be related to their volume and mitochondrial content, and therefore, the extent to which mitochondrial respiration can maintain cytoplasm integrity.

Although untested, the findings described here suggest the possibility that global or focal differences in ATP levels between and within blastomeres may contribute to the spectrum of fragmentation phenotypes observed in cultured human. We have shown that cytoplasmic microtubules, including those emanating from the sperm centrosome in the pronuclear may be proximal determinants of mitochondrial distribution within the cytoplasm (Van Blerkom et al, 2001). Therefore, how mitochondria are spatially positioned with respect to the planes of cell division may determine the uniformity of mitochondrial segregation and inheritance from the 1-cell stage onward. Whether differences in microtubular organization at the earliest stages of human development are influenced by intrinsic differences between oocytes and their ability to support spatial remodeling of the cytoplasm after sperm penetration warrants further study. However, if mitochondrial distribution is related to differential ATP production between and within blastomeres, these differences could manifest as cell-specific responses and activities leading to a spectrum of phenotypes with different effects on competence. In the context of the findings of Leist et al (1997), fragmentation during the early stages of human development may also represent a continuum that includes necrosis and apoptosis at the extremes with oncotic-like processes in the middle. This could explain the suggested co-existence of necrotic and apoptotic blastomeres in the same cleavage stage embryo (Jurisicova et al, 1996), while for others, it may explain apparent restoration of normal morphology between days 3 and 6 of culture, and the transfer of stage appropriate embryos with demonstrable developmental competence.

In addition to their assumed importance in energy production during early development, mitochondria have other critical functions including regulation of differential ion concentrations within the cytoplasm. While a similar role has been suggested for human oocytes and early embryos (Sousa et al, 1997; Van Blerkom et al, 2002), it remains to be determined whether inheritance of a reduced mitochondrial complement from the oocyte (Reynier et al, 2001), or disproportionate segregation during cleavage could be associated with impaired ion buffering in certain blastomeres, that depending upon extent and intracellular location, could have potential effects on plasma membrane function or cytoplasmic stability. It is well known that alterations in ion homeostasis can change intracellular free Ca^{2+} and K^+ concentrations and directly influence whether cell demise occurs by apoptosis or necrosis (Yu et

al, 2001). As discussed here, the interpretation of morphology or morphodynamic processes as they may relate to possible mechanisms of cellular degeneration and death in the early human embryo can lead to different conclusions. In other somatic cells, however, elevated levels of intracellular free Ca^{2+} (calcium overload) is a primary factor in necrotic cell death (Choi, 1995) while comparatively smaller increases appear to mediate apoptosis (Yu et al, 2002). If a similar situation prevails in early human embryos, perhaps the degree to which mitochondria are able to participate in the regulation of intracellular Ca^{2+} homeostasis could be a proximal determinant of cellular stability, fragmentation, or the form of blastomere demise. Studies with mouse oocytes 'aged' in vitro have associated impaired regulation of Ca^{2+} homeostasis with fragmentation, the initiation of PCD, and apoptosis (Gordo et al, 2002). Support for a mitochondrial participation in oocyte fragmentation comes from the study of the FVB strain, which has an inherently high rate of apoptosis and fragmentation, in vitro. Perez et al (2000) showed that the insertion of approximately 5000 mitochondria derived from granulosa cells reduced the rate of spontaneous oocyte fragmentation from 70% to 36% after 24 hours of culture. While it remains to be determined whether oocyte aging and fragmentation in the mouse is associated with forms of mitochondrial dysfunction or deterioration that could effect the regulation of cytosolic ions such as Ca^{2+} (Loew et al, 1994), it should be noted that comparable rates of fragmentation for in vitro aged human oocytes has not been described. Indeed, uninseminated and unfertilized, cumulus enclosed and denuded, MII human oocytes remain intact and largely unchanged during 4 or 5 days of culture (Van Blerkom and Davis, 1998). If a mitochondrial association with certain patterns of fragmentation in early human embryos is confirmed, the underlying mechanism(s) responsible for this activity may need to consider mitochondrial functions that are not directly related to ATP generation. In this regard, the phenomenon of fragmentation may become less enigmatic if continued study confirms a relationship between specific phenotypes and intrinsic differences between embryos and blastomeres with respect to mitochondrial content, distribution and activity.

For clinical purposes, tacit assumptions that fragmentation is a manifestation of developmentally lethal defects such as chromosomal abnormalities are difficult to reconcile with the observed development of normal-appearing blastocysts with numerical chromosomal disorders including aneuploidy, triploidy, and chromosomal mosaicism (Clouston et al, 1997; Drury et al, 1998; Evsikov and Verlinsky, 1998; Sandalinas et al, 2001). Therefore, fragmentation per se, especially when determined by a single inspection should not be used as the sole criteria for embryo selection or "de-selection" as long as indications of progressive cytokinesis exist. Because fragmentation does not necessarily effect all embryos within cohorts,

it will be important to investigate whether blastomeres that undergo different forms of fragmentation do so because of intrinsic defects or as a result of unique sensitivity to in vitro conditions, or both. A potentially important question in clinical IVF is whether certain oocytes may be predisposed to fragmentation after fertilization. Current evidence strongly indicates that follicle-specific conditions that occur during the preovulatory period, such as those associated with perifollicular blood flow (Van Blerkom, et al, 1997; Bahl et al, 1999; Huey et al, 1999; Gregory, this volume) and intrafollicular physiology and biochemistry (see review by Van Blerkom, 2002), are significant determinants of human embryo performance in vitro and competence in vivo. If extrinsic factors to which the oocyte may be exposed during the terminal stages of oogenesis have adverse influences at the molecular and cellular levels, their identification and characterization may provide new insights into the origins and mechanisms of fragmentation in the embryo, including those forms that are developmentally benign or lead to demise.

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CHAPTER FIFTEEN

BLASTOCYST TRANSFER UPDATE: PROS AND CONS

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INTRODUCTION

The aim of clinical in vitro fertilization (IVF) is to obtain a viable pregnancy leading to the birth of a healthy baby. This has been achieved through the transfer of embryos at the pronuclear (day 1), cleavage (day 2-to-3) and blastocyst stages (day 5-to-6). In fact, the transfer of a blastocyst established the first clinical IVF pregnancy (Edwards et al, 1995). However, with respect to outcome, whether better results are associated with uterine transfer at a particular stage or time (day) after insemination remains controversial. This chapter reviews current findings on blastocyst transfer and defines indications, advantages and disadvantages for transfer at this stage of preimplantation embryogenesis.

The development of the human embryo in vitro is generally characterized by retarded growth rates and loss of competence (viability), much of which has been attributed to chromosomal abnormalities (Munné and Cohen, 1998). Developmental arrest can occur at any time during the preimplantation stages and complete activation of the embryonic genome is crucial for development beyond the early cleavage stages. The initiation of transcription is an essential event in the course of early embryogenesis as it defines the transition of development driven by mRNA inherited from the oocyte to messages derived from the embryonic genome (Ménézo et al, 1998). Any delay in embryonic genomic expression will result in a decrease in the level of mRNA below a critical threshold required for development to progress. In this regard, the normality of development may well depend upon the system and conditions used to support embryogenesis through the preimplantation stages, and in this regard, the following approaches have been developed for the culture of

human embryos in general and to enhance the potential for blastocyst formation in particular.

THE CO-CULTURE SYSTEM

It has been suggested that the co-culture on a monolayer of somatic cells may have beneficial affects on embryo performance in vitro leading to the transfer of embryos with higher competence as compared to their counterparts cultured in cell-free (i.e., static) conditions. Different established cell lines have been reported to support embryo development to the blastocyst stage, including Madlin-Darby bovine kidney cells (MDBK) and African green monkey kidney epithelial cells (Vero) (Ménézo et al, 1990). Oviductal epithelial cells of several species, fetal bovine uterine fibroblasts, human granulosa cells and endometrial cells have been used in different studies (Simon et al, 1999; Veiga et al, 1999a).

While the mechanism(s) by which co-culture may exert a beneficial effect is not well understood, two possibilities have been suggested. The first involves the removal of toxic components from the culture medium, such as heavy metal divalent cations and metabolic inhibitors. The second mechanism proposes the contribution of embryotrophic regulating compounds such as small metabolites and growth factors by the cellular monolayer. To date, no specific factor(s) that might promote or agent(s) that could retard development has been identified. It has also been suggested that in contrast to the static conditions associated with conventional IVF culture systems, potential benefits to embryo development result from their exposure to a dynamic or ‘conditioned’ environment created by the feeder cells. Interaction with metabolic pathways may also be an important aspect of co-culture, and it is likely that the metabolism of “feeder” cells generates small molecules that promote the occurrence of embryo metabolism and other biosynthetic activities at normal levels. The feeder layer may also contribute amino acids to the culture system which could lead to a better balanced in vitro environment, although it is noteworthy that certain amino acids can be toxic by virtue of catabolism-associated ammonium ion formation. The role of feeder cells may be related to a capacity to eliminate or recycle potentially toxic substances including enzymatic formation of alanine, glutamine and methionine. Co-culture also provides a good Red-Ox potential (equilibrium between reducing and oxidative substances in favor of reducing activity) which is necessary to avoid the generation of potential toxic reactive oxygen species by lipid oxidation (Ménézo et al, 1998).

The reported beneficial effects of somatic cells for embryo co-culture are neither tissue nor species-specific. Co-cultured embryos usually have higher cell numbers than their counterparts cultured in simple media (Vlad et al,

1996). The choice of medium for embryo co-culture is critical since it needs to fulfill the requirements of both embryo and feeder cells, and in this regard, several reports have described the use of feeder cells to generate blastocysts for transfer (Ménézo et al, 1990; Ménézo et al 1992a; Guerin et al, 1997; Rubio et al, 2000a). However, much of the interest and work in the area of co-culture has ceased owing to concerns related to possible iatrogenic exposure of human embryos to pathogenic agents in the feeder cell lines, especially those derived as primary cultures as opposed to well-established cell lines.

SEQUENTIAL MEDIA

It has become evident that the preimplantation embryo has stage specific nutritional requirements. In the mouse, for example, it has been shown that levels of carbohydrates and amino acids which permit development through the early cleavage stages cannot support blastocyst development, and vice versa. (Gardner, 1998a). While fertilization and early development can be achieved in a very simple medium, two different approaches have been proposed for human embryo culture (see Biggers this volume).

The first approach uses a very simple culture medium with amino acids and low glucose and pyruvate concentrations, and includes EDTA to chelate iron and copper ions that are potentially embryotoxic. This system is designed to allow the embryo to better control the regulation of metabolite reserves by not disturbing endogenous pools. (Ménézo, unpublished). The second approach utilizes a more complex medium containing the ingredients that are currently thought to be necessary to support important metabolic reactions. It has been suggested that because this approach may be more similar to *in vivo* situation and to the co-culture system (Ménézo et al, 1999a), it should eliminate the possibility of retarded cell cycles. From day 3 of culture, after complete embryonic genome activation has normally occurred, the embryo requires a more complex medium to support increased cellular activities. At this stage, growth factors such as insulin appear to become essential while EDTA has been shown to have a deleterious affect on further development. Glucose has been shown to be the most important nutrient for the human blastocyst (Gardner et al, 2001) and for those embryos that develop to the blastocyst stage, the uptake and utilization of glucose and pyruvate have been reported to occur at levels higher than in embryos in which development arrests.

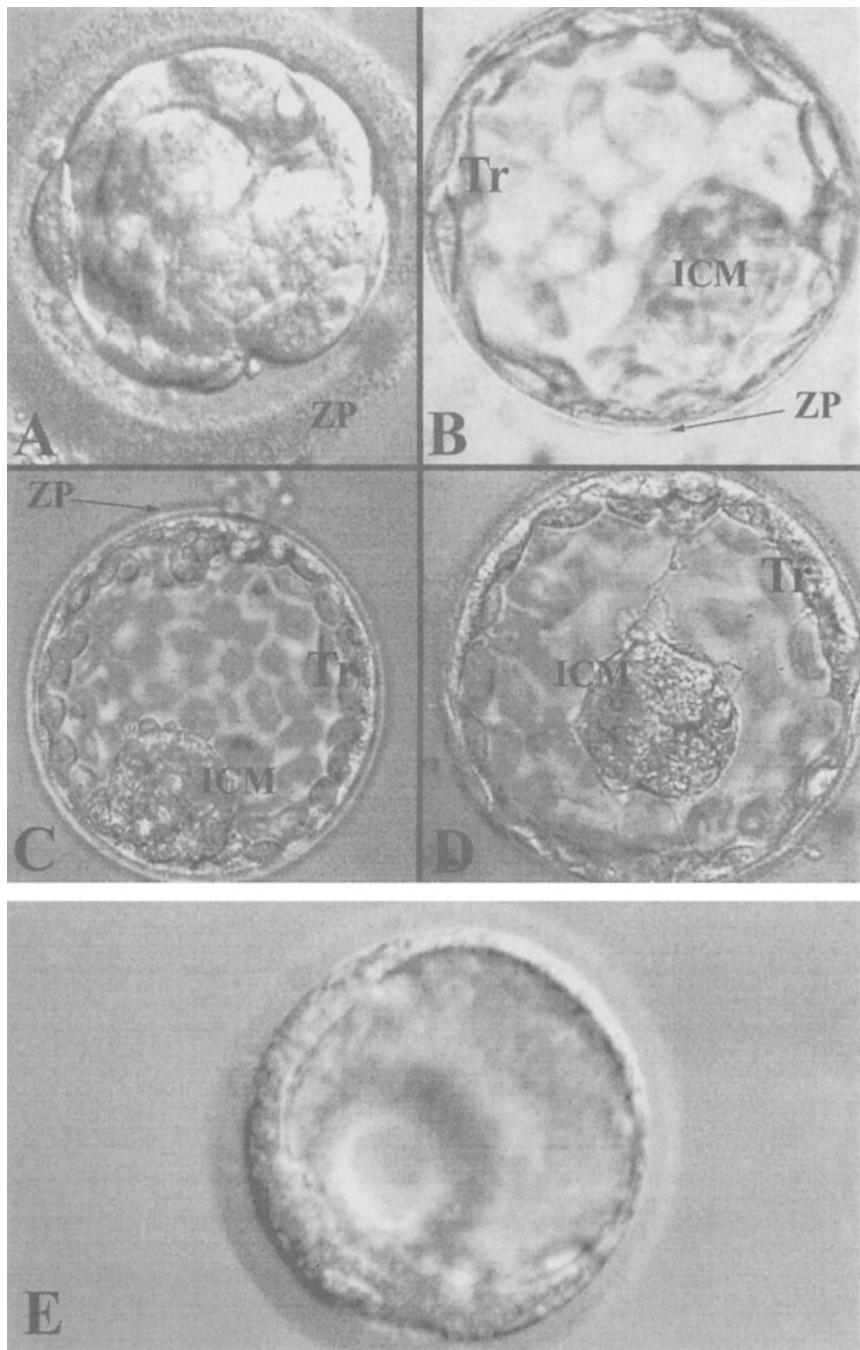
The morphology of the different embryo stages from fertilization to blastocyst has been well documented by numerous investigators (see Ménézo et al, 1999b). Compaction starts late on day 3 and by day 4, individual cells can no longer be distinguished. The first signs of blastocoel formation are evident late on day 4 (early cavitation) and by day 5 a distinct blastocoel and

the initial structures corresponding to the inner cell mass (ICM) and trophectoderm are evident. An expanding blastocyst has a clear ICM and trophectoderm and a thin zona pellucida (ZP) as shown in Figures 15.1A-C. A substantial increase in the embryo diameter is observed during expansion and the maximum diameter is attained when the fully expanded blastocyst has reached the limits allowed by the ZP. The hatching process is initiated when the trophectoderm starts to herniate through the ZP and when completed, an empty ZP is observed (Dokras et al, 1993; Fong and Bongso, 1998a; Gardner et al, 2000a).

In order to obtain satisfactory outcomes, it is essential to make detailed observations of developing embryos and blastocysts at high magnification and to replace only 'real' blastocysts characterized by a distinct ICM and well-structured trophectoderm (Fig. 15.1D). Structures without a defined ICM, a trophectodermal cell number that is stage inappropriate, as well as vacuolated embryos with contain no true blastocoel cannot be considered blastocysts and therefore should not be transferred (Fig. 15.1E). The presence of necrotic or degenerative areas within the ICM and trophectoderm should also be noted as these may have a negative impact on outcome. The apparent overall frequency of development to the blastocyst stage can be incorrectly increased if these 'pseudoblastocyst' structures that can occur within and between cohorts are inadequately classified by the IVF program. Recent studies show that blastocyst morphology correlates with implantation and pregnancy rates. Good quality blastocysts are associated with high implantation rates while the highest rates are observed with blastocysts in the process of hatching at transfer (Balaban et al, 2000). Richter et al, (2001) have demonstrated that blastocyst characteristics which have the highest impact on outcome are the size and shape of the ICM (Figure 15.1D).

PRONUCLEAR MORPHOLOGY AND BLASTOCYST FORMATION

There is evidence for a correlation between pronuclear morphology and the ability of the zygote to reach the blastocyst stage. Zygotes scored according to the distribution and size of nucleoli in each pronucleus were assessed for their potential to develop to blastocyst. It was found that those zygotes with equality between pronuclei (size, numbers and distribution of nucleoli) had a higher rate of blastocyst formation than those with unequal



FIGURES 15.1A-E. Morphology of blastocyst development

pronuclei (49.5% vs. 28%; Scott et al, 2000; Scott this volume). Balaban et al, (2001a) developed a different grading system for pronuclear scoring and described an “ideal” pronuclear pattern (so-called, 0 PN pattern). They claimed these embryos had a faster cleavage rate, resulting in day 3 embryos with good morphology and a 72% blastocyst rate compared with 12.7% development to blastocyst for zygotes with pronuclear “abnormalities.” In both studies, it was claimed that blastocysts developing from embryos having a good pronuclear score had a higher potential for implantation. Whether pronuclear morphology can be used to predict embryo competence at the blastocyst stage will require additional confirmation.

EARLY CLEAVAGE MORPHOLOGY (DAY 3) AND BLASTOCYST FORMATION

It has been clearly demonstrated that the rate of blastocyst development is associated with a number of clinical parameters such as maternal age, etiology of infertility, and paternal factors (Janny and Ménézo, 1994; Shoukir et al, 1998; Veiga et al, 1999). A number of studies have analyzed the relationship between embryo morphology on day 3 and the capacity of the embryo to develop to a blastocyst (Racowsky et al, 2000; Balaban et al, 2001; Langley et al, 2001). It is evident that an effective culture system for day 3 embryos is an essential pre-requisite for extended culture to the blastocyst stage. If the system is inadequate or the embryos have intrinsic defects impairing early development, blastocyst formation will be negatively effected. Blastocyst culture should therefore only be considered in IVF programs where culture systems provide good quality embryos on days 2-to-3. It has been reported that the number of blastomeres present at day 3 is related to the blastocyst formation rate (Janny and Ménézo, 1994; Veiga et al, 1999; Shoukir et al, 1998) with an increased frequency of blastocyst formation associated with rapidly cleaving day 3 embryos. However, Langley et al (2001) demonstrated that even those embryos with only a few cells on day 3 can form blastocysts that implant; indeed, some blastocysts developed from embryos at the 2-cell stage on at day 3 (7.1% blastocyst rate).

When correlations between blastocyst formation and the number of cells (Table 15.1) or the percentage of cytoplasmic fragmentation (Table 15.2) observed at day 3 are established, outcome results point to an increase in blastocyst formation with better embryo quality when derived from embryos with stage appropriate cell numbers or a lower percentage of fragmentation on day 3 (Institut Dexeus, unpublished data). These observations involved supernumerary embryos (from day 3 transfer) whose culture was extended with resulting blastocysts cyropreserved.

TABLE 15.1. BLASTOMERE NUMBERS IN DAY 3 EMBRYOS AND DEVELOPMENT TO THE BLASTOCYST

No of cells at day 3	No of embryos (%)	Frozen blastocysts	Blastocyst rate
< 4 C	16 (1.9 %)	0	0
4-6 C	525 (61.8 %)	206	39.2%
> 6 C	308 (36.3 %)	169	54.9%

(Veiga et. al., unpublished data)

TABLE 15.2. FRAGMENTATION IN DAY 3 EMBRYOS AND DEVELOPMENT TO THE BLASTOCYST

%fragments at day 3	No of embryos (%)	Frozen blastocysts	Blastocyst rate
<20%	577(68.0%)	272	47.1%
20-50%	269 (31.7 %)	103	38.3%
>50%	3 (0.3 %)	0	0

(Veiga et al unpublished data)

The association between slow development, fragmentation, multi-nucleation and a reduced potential for blastocyst development was reported by Alikani et al (2000). In another study of the predictive value of day 3 morphology on blastocyst formation on day 5, Rijnders and Jansen (1998) found a correlation between early embryo quality and rates of blastocyst formation, implantation and pregnancy after uterine transfer. They reported an overall blastocyst rate of 39%. However, when good quality embryos (adequate cleavage rate- 4 cells on day 2; 6-8 cells on day 3, no fragments or multinucleation) were considered, it reached 47%, while only 21% of poor quality embryos were able to develop into blastocysts. Of particular note was the finding that when data for blastocyst development and embryo transfer were examined, they found that only 51% of the embryos that were predicted to develop into blastocyst based on day 3 morphology actually did so, and it was these embryos transferred on day 5. Similarly, Graham et al, (2000) attempted to predict blastocyst development by assessing embryos on day 3.

In this study, only 48% of day 3 'selected' embryos developed to the blastocyst stage. These findings indicate that 3 morphology may have only limited prognostic value for subsequent blastocyst formation.

BLASTOCYST FORMATION AND MALE FACTOR INFERTILITY

The evidence for a strong paternal effect on blastocyst formation was suggested by Janny and Ménézo (1994). Using co-culture techniques, the authors determined the blastocyst rate in IVF patients in which insemination involved normal sperm, frozen donor sperm and sperm with impaired quality. While the blastocyst rate was between 40% and 50% for cycles where normal sperm were used, the rate was significantly decreased when frozen sperm and poor quality sperm were used (34.4% and 34.6% respectively) These investigators were unable to explain the origin of these differences but suggested they may be due to defects DNA, retarded pronuclear formation, and/or delays in the cell cycle at the time of genomic activation. Similar findings have been described by others and in one study, Jones et. al. (1998) reported that blastocyst rates on day 6 were significantly decreased in couples where no female factor could be identified, and in other studies, blastocyst rates were observed to be negatively effected in male factor patients with the magnitude of the decrease inversely related to the frequency and severity of the spermatogenic defect (Shoukir et al, 1998; Schoolcraft et al, 1999; Balaban et al, 2000, 2001b). These studies also confirmed reduced blastocyst development in these women and lower implantation rates than in nonmale factor IVF cycles.

The impairment in blastocyst development is especially evident when epididymal or testicular sperm are used (Balaban et al, 2001b). The lowest blastocyst rate is attained in couples where spermatozoa are obtained in instances of non-obstructive azoospermia. In these cases, the progression to the blastocyst stage is slower and the implantation rate negatively effected. In contrast, decreased implantation rates have not been reported when embryos are transferred during the early cleavage stages. While this finding indicates that in certain male factor cases the early embryo may be competent, it should not be regarded as conclusive evidence for a negative effect on blastocyst development as the outcome findings may also relate to culture conditions that are inconsistent with blastocyst development. Whether embryos from severe male factor cases may be competent to implant on day 3 but not on day 5 remains to be established.

BLASTOCYST DEVELOPMENT AND MATERNAL AGE

It has been reported that maternal age has a negative influence on blastocyst formation in general and pregnancy rate after blastocyst transfer in particular (Veiga et al, 1999a Langley et al 2001). While Janny and Ménézo (1996) reported lower blastocyst rates with increasing maternal age, other studies have not seen a similar association (Gardner et al, 2000b), despite the finding that implantation and pregnancy rates were lower with advancing age. Different parameters including ovarian reserve and oocyte quality, both of which may be related to the occurrence of chromosomal abnormalities, need to be considered when analyzing the effect of maternal age on blastocyst formation and implantation. Gardner et al (2000b) note that maternal age associated chromosomal abnormalities may not be incompatible with blastocyst formation, but would effect further development and consequently implantation. The majority of authors, however, report a reduced rate of blastocyst formation with advancing maternal age. This is often seen in conjunction with the development of low numbers of oocytes and consequently embryos. It would seem, therefore, that culture to the blastocyst yields poor results in advanced age patients.

BLASTOCYST DEVELOPMENT AND RESPONSE TO OVARIAN STIMULATION

Patients with a good response to gonadotrophins produce a higher number of oocytes and potentially more zygotes. A number of studies, however, have failed to show an absolute correlation between ovarian response and blastocyst development (Schoolcraft et al, 1999; Veiga et al 1999a). It is evident that the higher the number of oocytes, pronuclear (day 1) and 8-cell embryos (day 3) increases the potential for blastocysts on day 5 (Jones et al, 1998). It has further been suggested that the negative maternal age effect on blastocyst development may be mediated by the response to ovarian stimulation (Scholtes et al, 1998) with a good response giving good blastocyst numbers. Despite the lack of an absolute correlation between ovarian response and blastocyst formation, it is apparent that the probability of achieving blastocyst transfer is related to the number of oocytes retrieved and consequently day 3 embryos obtained.

COMPARISON BETWEEN DAY 3 AND DAY 5 TRANSFER

The central issue related to the analysis of embryo transfers at the blastocyst stage is whether it yields higher implantation rates than transfer of cleavage stage embryos on day 3. As early as 1996, a prospective randomized

study was performed comparing day 3 and day 5 transfer. The implantation rate after blastocyst transfer was shown to be significantly higher even where no sequential media or feeder cells were used (Scholtes and Zeilmaker, 1996). Additional studies have demonstrated the advantage of blastocyst transfer and in some reports, implantation rate per blastocyst was described as extraordinarily high, although this could largely be attributed to patient selection (e.g., Gardner et al, 1998b). Milki et al (2000) also claimed improved outcomes with day 5 transfers with a selected population of patients in which maternal age and the number of 8-cell embryos on day 3 (>3) were considered. While similar improved outcomes have been claimed for non-selected patient populations (Del Marek et al, 1999), other studies have not demonstrated any difference between day 3 and day 5 transfer either in selected patient cohorts (≥ 4 or more pronuclear embryos; Coskun et al, 2000; Karaki et al, 2002) or in unselected cohorts (Veiga et al, 1999a).

Racowsky et al (2000) examined the relevance of the number of 8-cell embryos on day 3 with respect to whether transfer on day 3 or on day 5 was preferable. They showed that where no 8-cell embryos were available on day 3, no pregnancies were attained despite blastocyst transfer on day 5. This supports the recommendation of Alikani et al (2000) that presumably poor quality embryos (stage-inappropriate with regard to cell number) should be transferred to the uterus as soon as possible. Racowsky et al (2000) were unable to demonstrate any differences in outcome when only one or two 8-cell embryos were available for transfer, with the exception of a reduction in the triplet pregnancy rate. The mean number of embryos replaced was 3.7 for day 3 transfers and 2.2 for blastocyst transfer. This is in contrast to data published by Balaban et al, (2001c), who showed that even when the blastocyst rate was low, such as with poor quality embryos (26.2%), the pregnancy and implantation rates were higher on day 5 than on day 3 (15% vs. 5.9%, respectively).

A review of current studies that compare transfer on day 3 or day 5 provides no conclusive data to clearly suggest an advantage for blastocyst transfer. However, data analysis is confounded by different criteria for patient selection in different studies (Garcia, et al, 2001). It has been suggested that the higher implantation rates from blastocyst transfers may be due to uterine contractility which could expel embryos transferred on day 2 or 3 (Fanchin et al, 2001). These investigators studied uterine contractility from the time of hCG administration through the luteal phase, and reported a significant decrease in contractions to a nearly quiescent state in the uterus 7 days after hCG administration (i.e. on day 5).

BLASTOCYST TRANSFER IN PATIENTS WITH PREVIOUS IMPLANTATION FAILURES

Transfer at the blastocyst stage has been applied with good results to poor prognosis patients, i.e., couples with repeated implantation failures, (Ménézo et al, 1992a; Olivennes et al, 1994; Guérin and Nicollet, 1997; Veiga et al, 1999a). All these studies, however, lack a control group. In a prospective non-randomized study published by Simon et al, (1999), no increase in the implantation rate was observed after blastocyst transfer in IVF patients with repeat implantation failures, as compared to outcomes with day 3 transfers. A significant increase was observed with blastocyst transfers in ovum donation cycles where previous donation cycles resulted in failure. This suggests that blastocyst transfer may improve implantation rates in patients with adequate uterine receptivity. No such improvement was observed in IVF patients in whom their own oocytes were used, which suggests impairment in uterine receptivity may be an important cause for implantation failure in these cases. In a retrospective cohort study (Cruz et al, 1999), patients with previous implantation failure were given the option of day 3 or day 5 transfer when at least 3 or more embryos at the 8-cell stage occurred on day 3. The results show clinical pregnancy and implantation rates significantly higher in the blastocyst transfer group. However, the number of patients in this series was too small to draw meaningful conclusions.

We (Veiga et. al., unpublished) have conducted a study to evaluate the different options for embryo transfer and their efficacy in patients with the following characteristics: (a) previous implantation failures and (b) ≥ 10 embryos replaced and/or ≥ 3 previous transfers without pregnancy (Table 15.3) Patients with more than four pronuclear embryos were randomized for transfer on day 3 with assisted hatching (AH, see Wright and Jones, this volume), transfer at the blastocyst stage as intact embryos, or transfer at the blastocyst stage with AH. As shown in Table 15.3, the best results were obtained with the replacement of blastocysts that were artificially hatched with a laser.

While this data is derived from a small number of IVF cycles, the findings tend to suggest that assisted hatching may promote blastocyst implantation.. Similarly, high pregnancy and implantation rates have also been reported in patients with poor prognosis after enzymatic treatment of the zona pellucida with the pronase (Fong et al, 1998b), which also suggests that optimization of contact between the endometrium and embryo may increase implantation. To achieve contact, the embryo must emerge from the encapsulating zona and any increase in pregnancy rate with AH may be due to the facilitation of embryo emergence.

TABLE 15.3. A COMPARISON OF THE OUTCOME FROM EMBRYO TRANSFER ON DAYS 3 AND 5 +/- ASSISTED HATCHING

	D3+AH transfer	Blastocyst transfer	Blastocyst transfer+AH
N. patients	22	22	23
N. transfers	22	19	20
X embryos replaced	3.2	1.9	1.8
N. pregnancies	5	6	13
pregnancy rate/patient	22.7%	27.3%	56.5%
pregnancy rate/transfer	22.7%	31.6%	65%
implantation rate	8.6%	18.9%	50%

BLASTOCYST TRANSFER AND MULTIPLE PREGNANCY RATE

The use of blastocyst transfer to avoid multiple pregnancies by transferring fewer embryos was proposed in the early 90's (Ménézo et al, 1992a; Olivennes et al, 1994; see Gerris, this volume). The high implantation rate obtained with blastocysts after co-culture or culture in sequential media allows the transfer of two blastocysts and the avoidance of higher order pregnancies (Gardner et al, 1998a, Milki et al, 1999). In younger patients, it may be acceptable to transfer only one blastocyst and would virtually eliminate the occurrence of multiple pregnancies (Del Marek et al, 1999). The advantage of day 5 transfer in patients with three or more 8-cell embryos on day 3 thus favors a reduction in the number of blastocysts transferred and multiple pregnancy rate despite the occurrence of monozygotic twinning, which can be a potential outcome (Racowsky et al, 2000). The occurrence of twins at high frequency in patients with more than 3 good quality embryos on day 3 is presently considered by some to be too high (see Gerris, this volume). In this regard, transfer of a single blastocyst may be considered in good prognosis patients (patients with >3 good quality embryos on day 3). The same is applicable when 'top quality' blastocysts (defined by an adequate trophectoderm and inner cell mass) are available for transfer (Gardner et al, 2000b) as blastocyst scores have been shown to be related to pregnancy and implantation rates.

A number of publications strongly recommend the transfer of two blastocysts in general, and one in good prognosis patients in particular, in

order to reduce the incidence of multiple pregnancies (Toledo et al 2000, Vidaeff et al, 2000; Garcia et al, 2001; Schoolcraft et al, 2001; Karaki et al, 2002).

CHROMOSOMAL ABNORMALITIES AT THE BLASTOCYST STAGE

We have demonstrated (Veiga et al, 1999b) that the percent of abnormal cells in arrested cleavage stage embryos (54%) is much higher than that observed in blastocysts (17.1%). The higher percent of normal cells found in blastocysts seems to support the hypothesis of 'self selection' against chromosomal abnormalities by extending culture to the blastocyst stage. This hypothesis is supported by the results from Evsikov and Verlinsky (1998) who showed that the degree of chromosomal mosaicism is lower in blastocysts when compared to cleavage stage embryos. It seems probable that developmental arrest during the cleavage stages will be higher if the abnormality involves genes that effect early embryo function. To date, no specific defects in gene expression that could adversely influence development during the cleavage stages have been identified in the human. Harper et al (1999) reported the frequency of chromosomally abnormal cells in blastocysts ranged from 7.5% to 86.6%, while Bielanska et al (2000) observed a rate of 16%, which was significantly lower than the 55% rate observed at the 2-cell stage. Coonen et al (2000) reported that the mean percent of diploid cells in human blastocysts was 72% while Sandalinas et al (2001) reported an aneuploid frequency of 19%. The study of Rubio et al (2000b) suggests that chromosomally normal embryos reach the blastocyst stage at a higher frequency than those with chromosomal abnormalities.

After analyzing the specific chromosomal abnormalities observed in human blastocysts, Sandalinas et al (2001) concluded that embryos with haploidy and autosomal monosomies (with the exception of monosomy 21) are eliminated during culture while trisomic embryos are not. However, other reports describe haploidy as well as monosomies in human blastocysts (Rubio et al, 2000b) and abnormalities in the ploidy of embryos were observed in blastocysts as well as in arrested embryos in our sample (Viega, unpublished). Triploid, tetraploid, and pentaploid cells have also been detected in blastocysts and arrested embryos at relatively low frequency (4-to-7%). While data from Clouston et al (1997) and Evsikov and Verlinsky (1998) showed similar rates (4.9%) of tetraploidy, Drury et al, (1998) described 4n signals for chromosomes X, Y, 13, 18 and 21 in seven expanded blastocysts at rates as high as 30%-to-40%. Polyploidy has been described as a normal feature in blastocyst development (Angell et al, 1987) and Benkhalfia et al (1993) reported the presence of hexaploidy in human morulae and blastocysts. It has been suggested that tetraploid trophectoderm cells may arise as a result of

of endoreduplication or endomitosis and could have a normal role in implantation (Drury et al, 1997). This phenomenon has been described by other investigators as well (Bielanska et al, 2000; Ruangvutilert et al, 2000 Sandalinas et al, 2001).

Chromosomal mosaicism has been detected in early human embryos (Munné and Cohen 1998) and has been shown to persist and to occur at high frequency at the blastocyst stage (Veiga et al, 1999b). Harper et al (1999) also reported a high mosaicism rate in human blastocysts whereas data from Evsikov and Verlinsky (1998) showed different results. The latter authors reported lower rates of blastocyst mosaicism when compared to early cleavage stage embryos. It has been reported that the percentage of mosaicism observed in the ICM is similar to the overall blastocyst mosaicism (around 10%). Data from Ruangvutilert et al (2000), Sandalinas et al (2001) and Bielanska et al, (2000, 2002) also point to a very high rate of mosaicism persisting to the blastocyst stage. It would seem that mosaicism could well be a normal event in human embryo development, especially polyplloid mosaicism, suggesting that the requirement for embryonic progression to the blastocyst stage may be associated with high ratio of normal/abnormal cells. In this respect, it may be the proportion of abnormal cells rather than occurrence per se that may determine competence.

BLASTOCYST TRANSFER AND MONOZYGOTIC TWINNING

An association between blastocyst transfer and monozygotic twinning when embryo culture involved sequential media has been recently reported by Ménézo and Sakkas (2002). Different studies with blastocyst transfer indicate a monozygotic twinning rate of 5% in ongoing pregnancies. Ménézo and Sakkas (2002) suggested that apoptosis may divide the ICM prior to the hatching process, which could lead to two separate ICMs and thus monozygotic twinning. In this respect, it is possible that cells of the ICM are more sensitive to apoptosis and less resistant to disruption than their trophectodermal counterparts. Alternatively, a 'deficient' culture environment may be the origin of this phenomenon.

BLASTOCYST FREEZING

The methodology for blastocyst freezing was described by Cohen et al (1985) and modified by Ménézo (1992b, 1996) uses a 0.2M sucrose solution containing 9% glycerol as a cryoprotectant (see Menezo and Geurin, this volume). The thawing of blastocysts is performed at room temperature with rehydration occurring in two stages (Ménézo and Veiga, 1997). The transfer of the surviving blastocysts is performed after a minimum of four hours of

culture following thawing, which allows the viability of the blastocysts to be assessed by their capacity to re-expand.

The freezing and thawing blastocysts obtained by co-culture with Vero cells resulted in a survival rate of 60-80% and a 20-25% pregnancy rate per transfer (Ménézo et al, 1992b; Veiga et al, 1999a). However, with the introduction of sequential media for blastocyst culture, survival after thawing was significantly reduced. It seems that the rapid elimination of cryoprotectant has a detrimental effect on blastocysts developed in sequential media and a slow thawing process is therefore advised such that the cryoprotectant is gradually eliminated in 8 steps as described by Cohen et al (1985). However, it is not always easy to determine if a blastocyst has survived after thawing as the most shrink due to loss of water from the blastocoel cavity. Even after several hours of culture, some will remain collapsed thus making it difficult to evaluate their potential competence. A 24 hour extension of culture permits a more definitive assessment of embryo morphology prior to transfer. In this respect, the synchrony between embryo and endometrium is not affected if blastocysts are thawed the day before the predicted time of transfer.

The importance of developing an effective method of blastocyst cryopreservation is clearly indicated if day 5 transfers are shown to improve outcome, either by better embryo selection ('self' or operator-selection by morphology) or uterine receptivity. If such advantages are confirmed and applied to limiting the number of transferred embryos to one or two depending upon patient characteristics, successful blastocyst freezing becomes a necessity in every clinical IVF program that adopts the day 5 transfer strategy. Another potential benefit of day 5 transfers relates preimplantation genetic diagnosis because it provides additional time to obtain the results of genetic analysis and assess embryo developmental potential. This has been recently demonstrated in preliminary results from our program with day 5 transfers of embryos biopsied on day 3, which has resulted in very acceptable pregnancy rates (42% pregnancy rate/transfer) and implantation rates (37.7% implantation rate).

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CHAPTER SIXTEEN

ASSISTED HATCHING IN CLINICAL IVF

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INTRODUCTION

One of the persistent mysteries in clinical in vitro fertilization (IVF) is why embryos that appear to be developing normally throughout the preimplantation stages fail to implant after uterine transfer. While it has been proposed that a certain proportion of failures may have a genetic or chromosomal basis, it has also been suggested that the inability of the embryo to emerge from the confines of the zona pellucida at the expanded blastocyst stage by a process commonly termed ‘hatching’ may render an otherwise normal embryo incompetent to establish a pregnancy owing to the persistence of a physical barrier between embryo and a receptive endometrial epithelium. The notion that the zona pellucida may be refractory to hatching is derived from observations of human embryos cultured in vitro through the blastocyst stages where some proportion of embryos within cohorts cultured in the same dish remain entrapped within the sibling blastocysts hatch and emerge completely. Although reasons why differential resistance to hatching may exist between embryos in the same and different cohorts is unknown, physical and biochemical differences in zona structure, zona hardening in response to culture conditions that are presumed to be suboptimal, and embryo defects which prevent an active participation in emergence have been suggested.

In order to enable hatching, methods that physically manipulate the zona pellucida have been developed to provide an opening through which the embryo can emerge if development progresses to the expanded blastocyst stage, a process commonly termed ‘assisted hatching’ (AH). Typically, AH is performed during the cleavage stages on embryos classified as high competence (see chapters by Gerris and Scott, this volume). Also, the application of AH generally assumes that no other intrinsic defects exist which may be associated with hatching failure. Although used in clinical IVF for over a decade, outcome results with AH have been controversial with some studies showing marked benefit while others show no benefit or in some cases, suggested to have a negative influence. This chapter reviews these

studies, the different methods of AH, and attempts to derive a critical 'consensus' regarding whether AH is beneficial with respect to outcome, and if so, under what circumstances and for which patients in particular should its application be considered.

THE STRUCTURE AND FUNCTION OF THE ZONA PELLUCIDA

The human zona pellucida (ZP) is an extracellular matrix of glycoproteins that surrounds the oocyte and preimplantation embryo. The murine ZP has been more extensively studied than the human, but both are structurally similar consisting of three glycoproteins whose roles in the mouse and human are assumed to be similar. One glycoprotein, ZP1, probably has a structural role, while the other two, ZP2 and ZP3, are involved in sperm binding and prevention of polyspermy (Talevi, 1997). The human ZP appears to have a bilaminar structure which can be seen upon exposure to acidic solutions where the most interior portion (facing the embryo) generally dissolves at a comparatively slow rate. This may be due to the differential arrangement of glycoprotein filaments that form the structure of ZP (Familiari, 1992). The human ZP is variable in thickness, ranging from 10 to 31 microns, with a mean dimension of 17.5 microns (Bertrand, 1995).

The role of the ZP in the preovulatory oocyte is to allow communication between the female gamete and the follicular environment by means of cellular processes from the corona radiata that remain fixed in position within the zona matrix and communicate with the oocyte by means of gap junctions (Rankin, 2001; Albertini, this volume). After ovulation, the ZP glycoproteins are involved in sperm binding with ZP3 associated with the acrosome reaction and after cortical granule exocytosis, biochemical alterations of ZP2 change the physical structure of the ZP resulting in a hardening of this acellular 'shell.' This so-called zona reaction provides a block to polyspermy by preventing passage of accessory sperm to the oocyte surface (Bauskin, 1999). The physical/chemical changes to the ZP that occur at the outset of fertilization are seen as an increased resistance to digestion by acidic solutions or proteolytic enzymes (see below) and by a thinning of the ZP from an average of 18.9 microns to 16.6 microns (Bertrand, 1995). After fertilization, the ZP is assumed to protect the developing embryo as it moves down the fallopian tube to the uterus. For example, the blastomeres of the precompaction cleavage stage embryo tend to be loosely associated (Dale, 1991) and in the absence of a protective layer, the potential exists for mechanical disruption of the embryo as it passes through the fallopian tube.

EMERGENCE OF THE EMBRYO FROM THE ZONA PELLUCIDA: BLASTOCYST HATCHING

The blastocyst emerges from the ZP in a process known as hatching and after full emergence, begins the implantation process. During the expanded blastocyst stage the ZP undergoes considerable thinning and normally is only a few microns in thickness when hatching occurs. The biochemical mechanism of hatching has not been extensively studied in the human but has been shown to be mediated by the embryo rather than by external factors. In the mouse, 'trypsin,' a trypsin-like protease is released by certain trophectoderm cells and has been proposed to be the zona lysin (Perona, 1986). Zona lysis does not appear to be highly conserved between species. For example, a cysteine protease that has no effect on mouse or human embryos is involved in the hatching of the hamster blastocyst (Mishra, 2000). It is also likely that highly motile filopodial extensions arising from a subset of trophectodermal cells provide mechanical forces that assist in the focal dissolution of the zona pellucida in the human (termed 'zona breakers' by Sathananthan, personal communication).

Assisted hatching is defined as an artificial breaching of the zona pellucida and was first introduced in clinical IVF by Malter (1989) and Cohen (1990) to improve clinical pregnancy rates by creating an artificial means for the embryo to escape. Originally, this approach was based on the observation that hatching rates of embryos cultured *in vitro* to the blastocyst stage were quite low (Cohen, 1985) and high implantation potential embryos could be identified either by an overall thin ZP or one that contained thinned regions as development progressed (Cohen, 1989a). Since the inception of mammalian IVF and embryo culture, suboptimal media and conditions have been ascribed to poor embryo performance and outcome. For the mouse, it has been long known that *vitro* culture conditions may alter in the structure of the mouse oocyte ZP resulting in failure of sperm penetration (DeFelici, 1982). These 'usual suspects' have also been implicated in the failure of human blastocyst hatching. However, given the fact that clinical IVF has been a successful treatment for infertility for nearly 25 years, this apparent iatrogenic defect must be embryo-specific. Partial zona dissection (PZD) was a precursor to current methods of AH and was originally used to facilitate fertilization in severe male factor treatment cycles prior to the introduction of intracytoplasmic sperm injection (ICSI). Successful outcomes with PZD demonstrated that introducing a breach in the ZP did not diminish implantation potential and was not associated with immunological complications for the embryo that were considered a possibility owing to a

direct exposure of a portion of the embryo to the uterine milieu (Cohen, 1989b). With continued use of PZD in clinical IVF, it appeared reasonable to believe that creation of an artificial rent in the ZP in IVF cycles involving normospermic cases might also improve outcome. This early realization led to the development of micromanipulation techniques that targeted the zona pellucida with the aim of enhancing hatching potential.

CURRENT METHODS OF ASSISTED HATCHING

MECHANICAL ASSISTED HATCHING

Mechanical AH can be achieved by PZD and this was the original method used by Cohen et al (1990b) where a micromanipulator-controlled glass microneedle is used to pierce the ZP, while the embryo is held in place under suction with a holding pipette. Typically, the microneedle is passed tangentially into the perivitelline space and the ZP is pulled away from the embryo slightly, after which the microneedle is passed out of the perivitelline space and through the ZP. The embryo is released from the holding pipette and remains “speared” by the microneedle. A slit is made through the ZP by massaging the ZP against the microneedle using the holding pipette. The resulting opening is approximately 20 microns in length.

ACID DIGESTION OR ZONA DRILLING

Acid digestion of the ZP makes use of the fact that the ZP can be dissolved in a low pH solution. Acidified Tyrode's solution (AT, pH of 2.2-to-2.6) has been used for decades to remove the zona pellucida in animal systems such as the mouse, and was used clinically to assist fertilization (Gordon, 1986) and remove blastomeres for preimplantation genetic diagnosis (Handyside, 1990). Typically, the embryo is held in place with a holding pipette while a glass micropipette with an internal diameter of 10 to 12 microns is placed against the ZP and the AT solution slowly expelled until a focal breach is observed (Cohen, 1991). If an embryo has a cluster of extracellular fragments or a local region where the perivitelline space is comparatively expanded, the zona rent is created in this area in order to avoid potential damage to blastomeres adjacent to the site of acid digestion. The caliber of the hole created by acid digestion is difficult to standardize as it is a function of the amount of solution deposited, the time required to create a breach (zona-specific resistance to dissolution), and in large measure, is often operator dependent. However, measurements of the hole produced by acid digestion indicate that with skilled operators, it is usually between 10 and 13 microns near the oolemma and between 15 and 18 microns at zona surface (Tao, 1997).

LASER ASSISTED HATCHING

Laser assisted hatching (LAH) or photoablation of the ZP has been used to facilitate fertilization and implantation in certain cases of male factor infertility or repeated implantation failures after IVF. For example, fertilization by incubation of oocytes in the presence of spermatozoa, sub-zonal insertion of spermatozoa by micropipet, and AH have used photoablation (Obruca, 1994). The ideal laser for AH provides photoablation with light at a wavelength not absorbed by DNA and will not produce heat. Typically, a system that conducts light to the ZP via fiber optic is attached to a micromanipulator (Feichtinger, 1992) and requires manipulation very similar to that used in acid drilling. The laser is aimed directly toward the embryo and penetration of the ZP is limited to 3 microns by use of a laser wavelength with a high absorption in water. Penetration of the ZP is achieved by progressively moving the fiber optic through the ZP which creates a hole, whose diameter is equal to that of the fiber optic thread, usually about 20 microns (Obruca, 1994). A number of different laser systems that use different sources and wavelengths have been proposed for clinical AH, each with unique energetic properties. The mode by which laser energy is delivered can also differ, for example, by transmitting laser energy along the optical path of the microscope objective. The advantage of this system is that no micromanipulator is required, and adjustment of the microscope stage moves the embryo and zona into the path of the laser (Neev, 1993,1995). The laser creates a tangential furrow through the ZP whose diameter is determined by focus of the laser beam. Complete rupture of the ZP often requires multiple firings of the laser with more of the zona moved into the laser path each time. Standardization of the rent diameter is one clear advantage of this method over acid or enzymatic drilling.

THE PIEZO-ELECTRIC MICROMANIPULATOR

A piezo-electric driver attached to a microneedle moves the needle longitudinally in a pulsatile fashion creating a motion that mechanically degrades the ZP. This operation is controlled by a micromanipulator and resulting debris can be removed from the ZP in a very precise fashion. (Nakayama, 1998)

ENZYMATIC DIGESTION OF THE ZONA PELLUCIDA

The protease pronase is used to digest the ZP of the blastocyst stage embryo on day 5 or 6 post retrieval. A solution of 0.5% pronase will dissolve the ZP completely in 1 to 2 minutes at 37°C. ZP dissolution is observed using

a dissection microscope and is halted by transferring the embryo to normal culture medium (Fong, 1998). This procedure is relatively simple as it does require a micromanipulator.

OUTCOMES WITH ASSISTED HATCHING

THE LAH METHOD

A retrospective study of 794 IVF/ICSI cycles that compared the four methods of AH described above was based on the availability of a particular method and the preference of the embryologist (Balaban et al, 2002). In this study, the results suggest that not only was there *no difference* between the methods of hatching but more importantly, implantation and pregnancy rates were similar between AH and the control groups (Table 16.1). While the results indicate that the method of AH does not have a major impact on clinical outcome, and while the investigators stressed that there were no changes in clinical or laboratory protocol during the study, this was not a prospective, randomized trial.

TABLE 16. 1 OUTCOME WITH DIFFERENT METHODS OF ASSISTED HATCHING

	Group 1 PZD	Group 2 AT	Group 3 Diode Laser	Group 4 Pronase	Group 5 Control
No. of ET cycles	239	191	219	145	188
Implantation rate	18.6%	17.4%	18.9%	19.1%	21.6%
Clinical Pregnancy rate	49.3%	46.0%	48.4%	46.8%	48.4%

(from Balaban et al, 2002).

Another study compared laser assisted hatching (LAH) techniques in patients meeting any one of the following criteria: (1) >37 years of age, (2) >2 previous IVF failures, (3) patients undergoing frozen embryo replacement and (4) women who were considered to be poor responders (Mantoudis et al, 2001). The study was a retrospective evaluation of 322 IVF cycles in which day 2 embryos were subjected to total LAH (a single hole completely through the zona, n=77), partial LAH (a single hole created without breaking the inner

membrane, n=158), or quarter LAH (thinning of one quarter of the zona without breaking the inner membrane, n=87). Clinical pregnancy rates in the total, partial and quarter LAH groups were 5.2%, 18.3% and 22.1% respectively. This data suggested that total LAH has a negative impact on implantation potential, a finding which contradicted an earlier study which concluded that the inner layer of the human ZP had to be fully breached in order to improve implantation rates (Tucker et al, 1993). Balaban et al (2002) did not find a detrimental effect associated with perforating the ZP with a laser, although this study AH was done on day 3 rather than day 2 (Mantoudis et al, 2001). If differences in sensitivity to LAH exist, they may be related to the type of laser or the energy delivered by different instruments.

MECHANICAL AND ACID DRILLING METHODS

The first use of AH in human IVF was described by Cohen et al (1990b) with 30 patients randomized into experimental and control groups. Fifteen women whose embryo transfers were on day 2 had 49 embryos subjected to mechanical AH or PZD. As a control, another fifteen women had 35 unmanipulated embryos replaced. In the AH group and control groups, implantation and ongoing pregnancy rates were 40% and 7%, and 25% and 6%, respectively. Although the authors claim that both implantation and ongoing pregnancy rates were *significantly improved* by AH, patients were not prospectively randomized and case characteristics such as number of embryos transferred or embryo morphology were not described or discussed. Trial 2 consisted of another 47 patients whose embryos were cocultured (CC) on either or two different bovine reproductive cell monolayers, followed by AH on day 2. Unexpectedly, AH in combination with CC seemed to have a negative effect on pregnancy rate when compared to the outcome with coculture alone, suggesting that the ZP does indeed serve as an important barrier between the embryo and its environment and under in certain conditions, breaching this barrier can have a negative impact on outcome. The patients undergoing AH in this study were given 100mg of tetracycline four times daily for four days and 16mg of methylprednisolone daily for four days after oocyte retrieval. The stated rationale for this treatment was that ZP rupture during the early cleavage stages might leave the embryo susceptible to infection by bacteria or to immunological attack by macrophages (Cohen, 1990b). This treatment regimen has been adopted by the majority of AH practitioners although its presumed utility has not been demonstrated in a prospective randomized trial.

Cohen et al (1992) reported outcome results from 3 AH trials that used acidic Tyrode's solution (zona drilling). In the first trial, 137 patients with normal FSH levels (less than 15mIU/ml at the time of ovulation induction) were randomly assigned to experimental and control groups. AH was

performed on 239 embryos (n=69) in the experimental group while the zona of 229 embryos was left intact in the control group (n=68). This trial showed *no significant difference* in either pregnancy or implantation rate per embryo replaced, with 53% control and 58% AH, and 21% control and 28% AH, respectively. In the second trial, 563 embryos from 163 patients were assigned prospectively to control or experimental (AH) groups. AH was applied selectively to those embryos defined as having poor prognosis for implantation using the following biometric parameters: (1) an average ZP thickness of greater than 15 microns, (2) less than five blastomeres on day 3 or (3) greater than 20% of the perivitelline space filled with extracellular fragments. In this trial, 186 out of 274 embryos were hatched with the remainder considered as controls. Transfers in this trial were therefore of a mixed variety consisting of intact and manipulated embryos. The reported pregnancy rate was significantly higher in the mixed group, 68% as compared to 48% in the unmanipulated group. The reported implantation rate was also higher, 25% compared to 18%. Improvements in outcome were attributed to the use of selective AH. Unfortunately, the study failed to provide definitive evidence for an AH benefit as the control group was not clearly defined and patients in the experimental group had both manipulated and non-manipulated embryos transferred. The comparatively high pregnancy rate observed in the control group also begs the question of whether these embryos were accurately classified as truly poor prognosis candidates.

A third trial included 30 putative poor outcome patients characterized by elevated baseline FSH levels (i.e. >15mIU/ml) at the time of ovulation induction. All patients were randomly assigned to experimental or control groups. Patients (n=15) in the experimental group underwent AH on 38 embryos, while 15 control group patients had a total of 41 unmanipulated embryos. All transferred embryos were either hatched or intact. The findings showed *no statistical difference* in pregnancy rates between experimental (53%) and control (33%) groups. However, the implantation rate per embryo transferred was significantly higher in the experimental group, 26% compared to 10% in the control group.

Since Cohen's initial reports of an apparent improvement in pregnancy rates following AH, there have been many studies have either corroborated or refuted the original findings. Some have included variables such as coculture, tubal embryo transfer (TET), and AH of thawed embryos, while others have sought to carefully define which patient population, if any, may benefit from AH. For example, a prospective randomized study by Hellebaut et al (1996) using PZD showed no effect on the implantation rate with unselected patients. In this study, 120 patients were assigned to AH or control groups using a 'minimization procedure' to control for patient age, number of embryos at transfer (ET), embryo quality and insemination method (conventional IVF or

ICSI). The pregnancy rate was 42% in the AH group compared to 38% in the controls, and implantation rates were similarly unchanged at 17.9 and 17.1%.

TABLE 16.2. SUMMARY OF OUTCOME FROM ASSISTED HATCHING STUDIES

Reference	Treatment	Number of subjects	Ongoing pregnancy rate	Per embryo implantation rate
COHEN ET AL, 1990	AH (PZD)	15	6/15 (40%)	12/49 (25%)
	Control	15	1/15 (7%) P=0.040	2/35 (6%) P=0.023
COHEN ET AL, 1992	i. AH	69	37/69 (54%)	67/239 (28%)
	i. Control	68	32/68 (47%) NS	49/229 (21%)
	ii. AH (selected)	80	41/80 (51%)	NS
	ii. Control	83	30/83 (36%) NS	70/278 (25%)
	iii. AH	15	7/15 (47%)	51/285
	iii. Control	15	2/15 (13%) P=0.05	(18%)P<0.05 10/38 (26%) 4/41 (10%) P=0.05
HELLEBAUT ET AL, 1996	AH (PZD)	60	42.1%	17.9%
	Control	60	38.1% NS	17.1% NS
SCHOOLCRAFT ET AL, 1994	AH (acid)	33	21/33 (64%)	40/122 (33%)
	Control	43	8/43 (19%)P=0.0001	12/185 (7%) P=0.0001
MAGLI ET AL, 1998	i. AH (acid)	45	14 (31%)	11.5%
	i. Control	42	4 (10%) P<0.05	4% P<0.02
	ii. AH	70	25 (36%)	15%
	ii. Control	53	9 (17%) P<0.05	6.3% P<0.01
	iii. AH	20	6 (30%)	11%
	iii. Control	18	1 (6%) NS	1.5% NS
SCHIEVE ET AL, 2000	AH	10703	33.9%	
	AH/non AH	3310	40.1%	
	Non AH	21490	33.3%	

A more recent prospective randomized study of poor prognosis patients was performed by Magli et al (1998). Poor prognosis was defined for women who (1) were older than age 37, (2) had three or more previous failed IVF attempts and (3) had both age and IVF failure factors. Patients from each group were prospectively randomized into study groups with no manipulation (i.e., controls) or AH. Both clinical pregnancy and implantation rates were significantly higher when groups 1 and 2 were compared to controls (Table

16.2). Group 3 appeared to benefit from AH, but the trend was not statistically significant. Maternal age, number of oocytes retrieved and number of embryos transferred were similar in the control and experimental groups in each of the three sets of patients. Collectively, the experimental group had a pregnancy and implantation rate of 33% and 13%, while the control group had a pregnancy and implantation rate of 12% and 4.1%, and these differences were *statistically significant*. This study is one of few that seem to show in a well-defined prospective randomized study, a beneficial effect of AH.

Analysis of the data from U.S. clinics submitted to the Society for Assisted Reproductive Technology (SART) for 1996 shows that AH was performed in 39% of the 35,503 cycles reported. The pregnancy rate in the 10,703 cycles (30% of the total) where only hatched embryos were replaced was 33.9%. In the 21,490 cycles where no embryos were hatched the pregnancy rate was 33.3%. There was an additional 3310 (9%) cycles involving both hatched and non-hatched embryos resulting in a pregnancy rate 40.1%. The pregnancy rates for hatched and non-hatched embryos were not significantly different, but as this data was not broken down into age groups or patient diagnoses, interpretation of results is problematical (Schieve et al, 2000). Still, this collective data does show that universal or unselected use of AH appears to have no clinical benefit, but in selected cases, such as patients of advanced maternal age, AH may be an appropriate procedure.

INDICATIONS FOR SELECTIVE ASSISTED HATCHING

Assisted hatching appears to have no clinical benefit for so-called good prognosis patients (see Gerris, this volume). In a study by Hurst et al (1998), embryo implantation rates were not significantly different between hatched and control groups; however, only 20 patients were included in this study (13 controls and 7 AH) which makes it difficult to draw a definitive conclusion. Other studies suggest that embryo implantation in poor prognosis patients may be improved by the application of AH. Poor prognosis patients are defined by one or more of the following characteristics: (1) advanced reproductive age (>39 yrs), (2) >3 failed previous IVF attempts, (3) elevated basal FSH levels (>15IU/ml) and (4) poor embryo quality defined by morphological features such as percent fragmentation, stage-appropriate, and uniformity of cleavage. Often these prognostic factors are inter-linked.

THE 'OLDER' PATIENT

A report of AH in women of advanced reproductive age by Meldrum et al (1998a) compared outcomes in this group with patients from previous years where transferred embryos were intact. The findings indicated increased embryo implantation rates from 14% to 21% in women aged 35 to 39 years,

and from 4% to 11% in patients 40 years and older. However, this study did not address other factors that could have contributed to an improved outcome, such as changes in ovarian stimulation protocols or modifications in embryo culture medium and techniques. While comparisons between experimental and controls which are not contemporary assume equivalence of study groups with no other confounding variables, definitive conclusions cannot be made from this report because the study size and design for control and experimental groups were not clearly defined.

A similar study, comparing experimental and non-contemporary controls examined pregnancy rates in poor prognosis patients after assisted hatching. A poor prognosis patient was defined as one ≥ 39 yrs, with "multiple" IVF cycle failures, and a day 3 FSH >10 mIU/ml (Schoolcraft et al, 1994). The control group consisted of 43 patients with the following parameters (averages): (1) age 37.2 ± 3.1 , (2) 0.91 failed IVF cycles (3) 4.3 embryos per patient and (4) FSH of 8.2 ± 2.3 . The experimental group consisted of 33 patients with the following characteristics (averages): (1) age 38.5 ± 3.3 , (2) 2.2 failed IVF cycles, (3) 3.7 embryos per patient and (4) FSH of 10 ± 2.8 . Not only are the control and experimental groups disparate in their case characteristics, i.e. age, number of failed IVF attempts, but they don't ever follow the proposed criteria of the poor prognosis patient. While the study claims to show an increase in pregnancy rate in the experimental group when compared to the control (64% vs. 19% respectively), the only conclusion that can possibly be drawn from this study is that assisted hatching does not decrease pregnancy rates. A larger randomized study of 385 patients with 211 receiving AH and 174 non-manipulated controls showed *no differences* in either pregnancy rates, 8.9% AH versus 5.1% control, or implantation rates, 3.75% AH, 3.55% control (Bider et al, 1997). However, pregnancy and implantation rates in the control group were well below outcome values routinely obtained in most IVF programs and therefore, the value of this data is questionable.

In a prospective randomized trial enrolling patients over the age of 35, *no significant differences* in outcome were found in the 41 patients where all embryos were hatched versus 48 control patients (Lanzendorf et al, 1998). Ongoing pregnancy rates were 29% versus 35% and implantation rates per embryo were 11.1% versus 11.3%. In these four studies of AH that involved older patients, one randomized trial showed no benefit of AH, while the three studies conducted using non-contemporary controls showed AH to be advantageous in older patients. There are two studies that were appropriate to the question of AH in older women. Lanzendorf et al, (1998) and trial 1 in the study by Magli et al (1998). The former study demonstrated no improvement in implantation or pregnancy rate after the use of AH, while the study by Magli et al showed a significant increase in pregnancy and implantation rate with the use of AH. Thus, the question of whether assisted hatching

counteracts zona dysfunction (if it exists) in older patients remains unanswered.

REPEATED FAILURE TO ACHIEVE PREGNANCY THROUGH IVF

A multicenter retrospective study compared outcomes between clinics that performed AH and those that did not (Meldrum et al, 1998b). Interestingly, in the 4,043 cycles that were evaluated, the clinics which offered AH had an overall higher pregnancy rate in their >40 yrs. patient population. This difference of course could have been associated with other factors such as the ability of a clinic to implement new culture technologies or AH techniques. Moreover, for patients with multiple IVF failures, AH did not alter the decline of pregnancy rate, indicating that while this procedure may improve outcomes for women of advanced maternal age, it should not be recommended or considered appropriate as a rescue technique for the repeat IVF failure patient.

INCREASED BASELINE FSH LEVELS

Studies have shown that basal FSH concentrations after down-regulation are predictive of the pattern of ovarian response to stimulation and of oocyte yield (Develioglu et al, 1999). In a study consisting of 30 patients with a baseline FSH of greater than 15mIU/ml, Cohen et al (1992) reported increased pregnancy (47% vs. 13%) and implantation rates (26% vs. 10%) in the 15 patients who received AH. This is a small study with preliminary data and patient parameters such as age and number of embryos transferred were not presented. Thus, recommending AH to this group of patients would be premature until additional studies are conducted.

ZONA PELLUCIDA THICKNESS

Measurement of ZP thickness on day 2 or 3 post oocyte retrieval has been a suggested indicator of embryo implantation potential (Cohen et al, 1989, 1992). It may be that a 'thick' ZP is more resistant to zona lysin, or that higher prognosis (i.e., quality) embryos have begun the process of ZP thinning somewhat earlier than their poor prognosis counterparts. A study by Cohen et al (1989) found a correlation between variation in zona pellucida thickness and pregnancy rate, where 40% (n=60) of the patients with >25% variation in ZP thickness became pregnant, while none (n=21) of the patients with <10% variation in ZP thickness became pregnant. Unfortunately the study does not provide information on patient age, diagnosis or infertility history. Thus, one cannot discern whether this phenomenon is associated with high basal FSH levels, age etc., or if it is a morphological anomaly which

should be identified in individual embryos and treated with assisted hatching. One question that needs to be answered is whether zona thickness and degree of 'hardening' as determined empirically by rates of acid dissolution are related. To date, there are no practical physical means that can be readily applied in the clinical IVF laboratory to quantify zona hardness. It may well be that a thicker zona may require more time to dissolve but does not present a barrier to hatching, while embryos with thinner zonae may have undergone aberrant structural changes that render this structure refractory to emergence. If procedures to quantify zona harness can be developed and easily applied in the IVF laboratory, associations with embryo performance (i.e. normality of hatching) after extended culture in optimized media would go a long way in providing embryo competence assessment with a the level of precision which could indicate which embryos may or may not benefit from AH.

A study by Bertrand et al (1995) investigated ZP thickness and clinical parameters. In seventy-five IVF cycles correlations between ZP thickness and patient age, duration of stimulation, cumulus maturity, number of retrieved oocytes, number of hMG doses, maximum E2 level and follicular volume were assessed. Among the relationships evaluated, ZP thickness was determined by statistical analysis (ANOVA) to be slightly influenced by maximum E2 level and the number of hMG doses. However, whether AH should be recommended or would benefit patients with an E2 value above a specific level or those receiving high doses of hMG could not be determined from this study.

THAWED EMBRYOS AND ASSISTED HATCHING

It has been suggested that the ZP may be altered after cryopreservation, resulting in failure of the embryo to escape due to iatrogenic ZP hardening (Tucker et al, 1991). A study of 65 thawed embryo replacement cycles using the PZD method of AH showed implantation rates for AH embryos to be 10/63 (16%) and control embryos 6/64 (9%). This difference was not significant due to low numbers (Tucker et al, 1991). In a later study by Check et al (1996), AH of 269 frozen thawed embryos from 79 patients involved drilling with acidic Tyrode's solution. The control group was comprised of matched patient pairs from an historical cohort. AH embryos implanted at a higher rate, 13.7% compared to 5.3%, and AH patients had a higher ongoing pregnancy rate, 28% versus 14%. However, Check et al (1996) noted that differences existed in the treatment protocols between the experimental group and the historical control group and therefore, concluded that it was not clear whether the apparent improvement in implantation rate was due to the changes in methodology or to assisted hatching.

Damage to the ZP of frozen thawed embryos has been investigated by Van Den Abbeel et al (2000), who found that the occurrence and extent of damage

(i.e., partial zona loss) was actually effected by the container in which the embryos were frozen, with plastic cryovials causing more damage than plastic mini-straws (16.6% versus 2.3%; $P < 0.0001$). Micromanipulation of these damaged embryos may have the potential to cause additional disruption, especially if suction is applied to the ZP by a holding pipette and the structure of the zona has been changed during cryopreservation. On the other hand, focal breaches in the ZP associated with cryopreservation may be viewed as fortuitous if they serve the same purpose as AH.

COMPLETE REMOVAL OF THE ZONA PELLUCIDA

A priori, one might think that if removal of small portion of the zona pellucida increases the implantation potential of an embryo, removal of the entire zona pellucida, especially at the expanded blastocyst stage, should have a larger impact on implantation potential. However, a prospective randomized study comparing enzymatic digestion of the ZP of blastocyst stage embryos with untreated controls showed *no difference* in either implantation or pregnancy rate (Isik et al, 2000). In 46 patients who had embryo transfers on day 5 or 6 post, 71 embryos from 24 patients were treated with 0.2% pronase to remove the ZP. A control group of 63 embryos from 22 patients was untreated. Ongoing pregnancy rates were 45.8% (11/24) in the treated group and 27.3% (6/22) in the control, rates that were *not statistically different*. Also, implantation rates were very similar, 19% and 24% in the control and treated groups, respectively. Participants in this study were good prognosis patients (mean age of 29.8 ± 4.5 years). This study is another example that questions whether AH is appropriate for this patient population.

A similar technique has been applied to cleavage stage embryos where instead of pronase, the ZP is removed by placing the day 3 embryo into a solution of acidic Tyrode's solution until complete dissolution of the ZP is observed, followed by culture in normal medium (Mansour, 2000). Two clinical trials were reported in this study, the first of which involved 52 patients under 40 years of age undergoing their first ICSI treatment. The second trial included 71 poor prognosis patients as defined by age 40 years or greater, and at least two previous failed IVF attempts. In the first trial the average age and embryo number in the control and experimental groups were 32.1 ± 2.5 , 3.2 and 33.2 ± 1.4 , 3.0, respectively. The 2 groups were comparable in terms of history, fertilization rate and total number of embryos, and embryo morphology, as indicated by a 3-teered scoring system. The results indicated that clinical pregnancy rates were *not significantly different* between the two control (10/25:40%) and experimental groups (12/27:44%). Thus, it was concluded from the first trial that complete zona removal with AH was of *no benefit* in this group of patients. In the second trial, the pregnancy rates were 7.3% (7/30) and 23% (3/41) in the control and treated

groups, respectively, and this difference was *statistically significant*. While the control and treated groups of patients were comparable, the number of embryos transferred per patient was not indicated, which makes it difficult to confirm any benefit of complete zona removal in poor prognosis patients. What is apparent however, is that zona free day 3 human embryos can survive and develop in the uterus. Nevertheless, directly exposing the entire embryo to the acidified solutions can have detrimental consequences and is almost certainly a technique that is highly dependant on practice, timing and skill. In this respect, it would seem prudent to suggest that it not be implemented as a routine procedure unless retained viability is established for each operator.

EXTRACELLULAR FRAGMENT REMOVAL

In order to minimize damage to blastomeres with acid drilling in fragmented embryos (see Van Blerkom, this volume), the solution is preferentially applied to the region of the ZP adjacent to fragment clusters. Once the ZP is ruptured, some or all of extracellular fragments can be removed by aspiration with a micropipette. In one preliminary study (Alikani et al, 1999), this procedure was reported to increase the implantation rate by 4%, but this development was described by the authors as a trend rather than an improvement with demonstrable statistical significance. The proposed benefit of fragment removal was suggested to result from the elimination of potential barriers to intercellular contact and communication required for compaction, or perhaps by preventing subsequent damage to healthy blastomeres if fragment degeneration is accompanied by the release of toxic agents. However, depending on the extent of fragmentation, the method of fragment removal itself can damage up to 11.4% of the manipulated embryos. Again, this is a technique that requires a high degree of skill and training, not to mention an abundance of time and staff available to perform such a procedure. Thus, unless additional and rigorous studies clearly demonstrate a statistically significant improvement in outcome in a specific patient population or class of embryos, routine use of this technique may be of no benefit or in some cases have negative consequences.

TWIN PREGNANCIES ASSOCIATED WITH ASSISTED HATCHING

As early as 1986, Edwards et al showed that IVF is associated with an increased risk of monozygotic twinning when compared to the incidence in spontaneous pregnancies of approximately 1.3%. Monozygotic twinning is also more common after ovulation induction, and in a Belgian study, monozygotic twins in twin and triplet pregnancies occurred at a rate of 0.45% in the general population and at 1.2% in treated patients (Derom, 1987). Manipulation of the ZP in mouse and human embryos by PZD or AT

digestion leads to abnormal hatching of the blastocyst stage embryo. Because the diameter of the hole is smaller than the one created during natural hatching, if the embryo emerges through a smaller than normal rent, 'pinching' of the trophectoderm and the inner-cell-mass (ICM) can occur, as evidenced by an hourglass configuration observed during hatching *in vitro* (Malter et al, 1989). It has been suggested that an abnormal or constrained hatching process might lead to monozygotic twinning, monoamniotic monozygotic twinning and potentially conjoined twinning, if complete separation of the ICM does not occur. A survey of U.S. IVF clinics performing ZP manipulation for assisted fertilization and AH showed 4 out of 143 (2.8%) pregnancies were monoamniotic monozygotic twins. This is higher than the 0.35 to 0.40% rate in the general population (Slotnick and Ortega, 1996). This survey also reported a monoamniotic twin from non-zona manipulated IVF cycle, although overall pregnancy rates in this group were not reported.

Two studies of AH have reported increased rates of monozygotic twinning (Hershlag et al, 1999; Alikani et al 1994). After AH, 8 out of 226 pregnancies (3.5%) included a monozygotic twin as compared to none in a control group of 122 pregnancies derived from intact embryos (Hershlag et al, 1999). Alikani et al (1994) showed that in cases where the ZP was manipulated in some way (ICSI, sub-zonal insemination, or AH), 6 out of 737 (0.8%) pregnancies included a monozygotic twin, though only 3 of the six cases actually had AH, resulting in a monozygotic twinning rate of 0.41%, which is comparable to that which occurs in *in vivo* conception (0.42%) (Bulmer, 1970).

Analysis of data compiled from U.S. IVF clinics for 1996 that reported results from 35,503 embryo transfers indicates that overall, 0.20% of transfers resulted in a monozygotic twin pregnancy. In 10,703 embryo transfers involving AH, monozygotic twin occurred at a frequency of 0.33% compared to 0.13% in 21,490 transfers where AH was not performed (Schieve et al 2000). The increase may be statistically significant but the incidence is very low. Sills et al (2000) has suggested that the increase in monozygotic twin pregnancies seen in IVF cycles occur as a result of multiple embryo transfer.

In a retrospective study of 3,546 embryo transfers, 23 of 1911 pregnancies (1.2%) were monozygotic twins. Sills et al (2000) assumed a natural monozygotic twin rate of 0.42% (Bulmer, 1970) and also assumed this to be a per embryo rate, because only a single oocyte is typically ovulated in natural cycles. Therefore, if multiple embryos are transferred, as in this study where the average was 3.2 per transfer, an expected increase in the frequency of monozygotic twinning rate would also be expected to increase by a similar amount, or 1.3%, which is similar to the observed 1.2% (Sills et al, 2000). Therefore, based on the current available data, it is difficult to confirm any association between AH and monozygotic twinning.

ZONA PELLUCIDA THINNING

A variation on AH is partial ZP thinning where the outside surface is affected but the structure is not ruptured. The reported advantage of this manipulation is that the ZP remains intact while the embryo remains protected from potential trauma associated with transfer. Initial studies of this method used mouse embryos because it has been shown that in vitro culture of mouse oocytes and embryos in certain media leads to zona hardening and impairment of blastocyst hatching (DeFelici et al, 1982). Cruciate thinning of the ZP (CTOZ) using acidic Tyrode's solution attempts to simulate the natural thinning of the ZP by application of the solution to the ZP in a biaxial direction, thus establishing a 'cruciate' area of thinning, although the exact size of the thinned area was not described. Hatching of blastocysts by day 5 in vitro occurred earlier and at a higher frequency in thinned embryos (n=175), as compared to untreated control embryos (n=165) (74% vs. 66%. Tucker et al (1993) used CTOZ in a prospective trial with 218 patients and reported a pregnancy rate of 45% (49/ 110) with treated embryos as compared to 37% (40/108) in the untreated or control group. This difference was *not statistically significant*, indicating that while cruciate thinning may enhance hatching in-vitro in the mouse, this method appears to have no obvious benefit in clinical IVF.

IS ASSISTED HATCHING OF CLINICAL BENEFIT IN IN-VITRO FERTILIZATION?

The above findings lead to the question of whether the design and implementation of clinical trials related to AH have been of sufficient specificity to provide a definitive role for this procedure as a general or patient-related adjunct in IVF. It is often the case in clinical IVF that technologies have been introduced without a full understanding of the biological processes that may be involved, and in some instances, without any preliminary studies in animal systems. A prime example is ICSI, which rapidly achieved universal acceptance for the treatment of male factor infertility. Many investigators cite the difficulty of providing the best available clinical techniques based on findings from truly randomized prospective studies. Assisted hatching has been more rigorously studied in comparison, but its effects and efficacy remain incompletely defined. To date, clinical studies have not included sufficient patient numbers and characteristics to demonstrate that AH is beneficial in all or selected patients.

A large multi-center randomized and prospective study has the potential to demonstrate whether AH is beneficial, and if so, in what instances (e.g.,

thawed embryos, thick zona) and for what patients (age, history, etc). However, the practice of clinical IVF is driven by the patients' desire to conceive and the IVF program's desire to present to the community high pregnancy rates. In many respects, these imperatives coincide such that IVF practitioners offer procedures suggested to be state of the art before efficacy is actually determined. Consequently, it seems unlikely that the type of multicenter or multinational study of AH that would be necessary to establish clear benefits will be undertaken.

Cohen et al (1990) suggested that AH increases implantation rates by allowing earlier exposure of embryonic trophectodermal cells to the endometrium. AH embryos have been reported to implant an average of 18 hours earlier than intact siblings, as measured by the onset of endogenous human chorionic gonadotropin (hCG) production relative to the day of oocyte retrieval (Liu et al, 1993). In this study of 149 AH patients, 72 clinical pregnancies occurred, while 57 clinical pregnancies resulted in the control group ($n = 151$). These results were *not statistically different*. In this study E2, progesterone and hCG were measured on Days 5-to-15 (post ovum retrieval) to establish the approximate implantation day. In both groups implantation was presumed to occur between days 7 and 13 post retrieval, but based on E2, P and hCG levels in luteal serum samples, control embryos were estimated to implant on Day 9.72 ± 1.47 , while AH embryos were estimated to implant on Day 8.96 ± 1.14 . This difference was *statistically significant* suggesting that implantation might be expected to occur earlier in AH embryos than in controls, since the man-made breech in the ZP allows for earlier hatching. However, because, Liu et al (1993) measured hCG levels in patient serum each day, it is impossible to determine precisely when implantation occurred or to designate a particular 24 hours interval during which an embryo actually implants. Also, the relevance of implantation timing to the second decimal point is questionable as it could be assumed that implantation can be timed to the minute. Furthermore, hCG levels in ongoing IVF pregnancies can vary greatly between normal embryos and between patients during the first weeks of pregnancy, and the relevance of hormone levels with respect to when implantation actually began can be confounded with multiple embryos transfers. In this instance, higher initial hCG levels may occur in the first few measurements, but the rate of increase and the relative level may change if one or more implanted embryos arrests.

The report that implantation in the control group was presumed to occur over a 6- or 7-day period suggests that early implantation may not be an advantage in the establishment of pregnancy. If a relatively short implantation window exists, and in particular women embryo development is asynchronous with uterine receptivity, implantation and pregnancy rates may be favored by transferring both AH and non-AH embryos. In this scenario, AH embryos would hatch before non-AH embryos, therefore theoretically increasing the

time that hatching embryos would be available to encounter a receptive endometrium. This may explain the increased pregnancy rate noted by Schieve et al (2000) in AH/non-AH embryo transfers when compared to transfers involving either AH or intact embryos. However, the logic of replacing treated and untreated embryos in IVF cycles seems difficult to reconcile with the notion of AH as a direct treatment of zona defects that at the least, ensures a possibility for emergence. It also runs the very real risk of creating higher order gestations, an outcome in clinical IVF which most responsible infertility programs go to great lengths to avoid (see Gerris, this volume).

AH has been suggested in some studies to increase implantation and pregnancy rate (Schoolcraft et al, 1994) and in others to have little or no effect (Hellebaut et al, 1996; Edirisinghe, 1999). The most effective method of AH used in trials with comparatively large patients numbers seems to be zona drilling using AT (Cohen et al, 1992). In contrast, published studies of LAH have involved relatively lower patient numbers and have largely been designed to show the efficiency of specific laser systems in a clinical setting, rather than to prove the benefits of AH. It is not evident which, if any, patients could benefit from AH. For example, in patients whose embryos have thinner zonae pellucida, AH has been shown to be detrimental (Cohen et al, 1996). Other patient subsets might also suffer from the effects of AH, such as those whose embryos have no hatching impairment or impediment.

Indeed, it seems probable that optimized culture conditions (see Biggers, this volume) may provide a greater improvement in pregnancy and implantation rates than AH (Cohen et al, 1990; Mercader et al, 2000). Embryos exhibiting spontaneously thinned ZP on day 3 appear to have greater developmental competence than those with a ZP that remains unchanged. The idea that suboptimal culture conditions can affect spontaneous hatching is borne out by experience with the so-called mouse' anti-hatching model' where embryos cultured in the absence of serum hatch at lower rates, 12% versus 87%, than those cultured with serum (Alikani et al, 1992; Schiewe et al, 1995). However, the value of this system as a model for the human is questionable as in the absence of serum or protein supplements, *in vitro* performance of mouse embryos during the preimplantation stages can be compromised.

As the biochemical requirements of the cultured human embryo are better understood and if clearly related to improved outcome (Van Langendonck et al, 2001), AH may be found to be largely unnecessary and restricted to only those cases of repetitive failure with embryos classified as normal at the blastocyst stage. But what of those embryos which fail to hatch, can they be presumed to be developmentally normal and should AH be applied in these cases (see Cummins, this volume)? Perhaps the more relevant issues relate to the mechanism of hatching and the need to better understand the actual

biology and chemistry of this process in order to identify the cellular activities and molecules involved. This could lead to methods for detection of specific factors and a deeper understanding of whether their absence has broader meanings for developmental competence.

In summary, no biometric methods have been developed or proposed that could clearly identify individual embryos that might require or benefit from AH. Certain patient populations such as those classified as poor prognosis have been suggested to benefit from AH, but at present, there is no conclusive evidence to support this contention. There are also risks to the embryo associated mechanical manipulations and potential toxicity from chemical, enzymatic and laser treatments. Good laboratory technique and operator training can reduce the potential for iatrogenic damage, but they may not be entirely eliminated. From the clinical evidence available to date, we conclude that AH has yet to be shown to have clear benefit in the treatment of human infertility. This is in part because (a) patients undergoing or selected for AH differ between IVF programs, (b) there have been no well designed, large scale prospective studies to determine efficacy and (c) comparative outcome data between groups is often of little value because of different methods of AH and very different pregnancy rates with designated control groups. We suggest that clinics offering AH should evaluate the rationale for performing the procedure and the accuracy of information they present to patients about whether or not it may be beneficial in their particular circumstances. To include AH in the routine IVF protocol simply to cover 'all possible bases' for the patient may be an acceptable rationale to some, but given the data reviewed in this chapter, may not be the most thoughtful. While AH is not the universal magic bullet once proposed for IVF failures, it may have a place in certain instances of infertility treatment. The unresolved issue and task at hand is to determine what that place may be.

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CHAPTER SEVENTEEN

PROSPECTS FOR OBTAINING VIABLE OOCYTES FROM CRYOPRESERVED OVARIAN TISSUE

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INTRODUCTION

As survival rates of patients with malignant diseases improve, the total and often irreversible sterility caused as a side effect of chemotherapy and abdominal irradiation assumes a greater significance. The sterilizing effects of cancer treatments may occur both through the loss of normal uterine function and through the partial or complete destruction of the primordial follicle reserve. Even if ovarian failure does not occur immediately, there is a substantial risk of premature menopause, especially in those aged over 30 years Whitehead et al, 1983; Birch et al, 1988). This loss of fertility is of particular relevance to young women and girls who are diagnosed with cancers when they are too young to have either started or completed their families. The childhood/youth cancer types whose treatments place females at the greatest risk of ovarian failure after chemotherapy, radiotherapy or bone marrow transplantation, but for which patients have high survival rates, have recently been reviewed by Oktay and Yih (2002). These include leukaemia, neuroblastoma, Hodgkin's lymphoma, osteosarcoma, Ewing's sarcoma, Wilms tumour and non-Hodgkin's lymphoma. High-dose chemotherapy is also being used for an increasing number of non-malignant conditions such as autoimmune diseases and thalassaemias. The risk of premature loss of ovarian function after exposure to these agents is further increased in older patients who naturally have a reduced ovarian reserve, and is dependent on the type of chemotherapy agent or dose of radiation used to treat the cancer.

The options for preserving the fertility of young oncology patients range from no medical intervention at all to the use of invasive procedures to harvest tissues or isolated cells (Otay et al, 1998; Picton et al, 2000). Each method has its own advantages, disadvantages and risks. Assisted reproductive techniques together with cryopreservation enable collection and storage of germinal vesicle (GV) or metaphase II (MII) oocytes and more practically, embryos after in vitro fertilization (IVF). A radical alternative strategy to oocyte and embryo freezing which can be used by

young girls as well as adults is the cryopreservation of ovarian tissue. This approach has the added advantage that it may also provide a means of conserving the fertility of young women with a familial history of premature ovarian failure. Although not without its criticisms, compared with the ethical dilemmas of embryo cryopreservation and the technical problems of freezing mature oocytes, ovarian freezing represents an attractive general strategy because it completely removes germ cells from exposure to harmful cytotoxic agents and it offers the potential to restore natural fertility by autografting the thawed tissue at an orthotopic site.

OVARIAN TISSUE CRYOPRESERVATION

Both the architecture of the human ovary and the biology of primordial follicles make ovarian cryopreservation possible. The ovarian cortex of a young human ovary is packed with hundreds of thousands of quiescent primordial follicles that represent the reserve from which all antral follicles and fertile oocytes will ultimately develop (Faddy et al, 1992). Thus, a thin sample of the cortical region of the ovary obtained either by laparoscopy (Meirow et al (1999), laparotomy or oophorectomy, can yield large numbers of primordial and primary follicles. In theory, primordial oocytes are better suited to surviving cryopreservation than secondary oocytes as they are smaller, undifferentiated, lack a zona pellucida and cortical granules, are relatively metabolically quiescent and perhaps most importantly, are by far the most abundant stage present at every age (Table 17.1). Additionally, primordial follicles are apparently more tolerant than mature oocytes to insults such as immersion in hypotonic cryoprotectant solution and cooling to very low temperatures, as they are smaller and have more time to repair sublethal damage to organelles and other structures during their prolonged growth phase. Indeed, human (Hovatta et al, 1996; Newton et al, 1996; Oktay et al, 1997), marmoset monkey (Candy et al (1995) and mouse Gosden, 1990; Harp et al, 1994; Carroll and Gosden, 1998) primordial follicles have all successfully survived cryopreservation to liquid nitrogen temperatures.

THE PRINCIPLES AND PRACTICES OF OVARIAN CRYOPRESERVATION

The first serious attempts to cryopreserve ovarian tissue and restore endocrine function were carried out in rodents in the 1950s (Parkes and Smith, 1953; Deanesley, 1954; Parkes, 1956,1957). At this time, ovarian tissue that had been frozen in a mixture of glycerol and physiological saline and subsequently autografted onto a subcutaneous site was shown to restore oestrous activity in ovariectomized rats (Green et al, 1956). Follicle survival rates in these grafts were, however, low. By 1960 the successful isografting of frozen-thawed murine ovaries onto the ovarian

bursa had lead to the restoration of natural fertility and the birth of healthy pups Parrot, 1960). Despite this initial success, the technology required to support further progress was not available and the work was largely abandoned. However, with the advent of assisted reproduction technology and attendant research into freezing of spermatozoa (Agca and Crister, 2000) and embryos (Trounson and Dawson, 1996; Byrd, 2002;

TABLE 17.1. COMPARISON OF THE CHARACTERISTICS THAT INFLUENCE CRYO-SENSITIVITY AND SUITABILITY FOR CRYOSTORAGE

Material	Primordial oocyte	Full-size immature (GV stage) oocyte	Full-size mature (MII stage) oocyte
Availability	Abundant, always present	Scarce, only from antral follicles	Scarce, only at mid-cycle
Ease of collection	Easy, e.g. biopsy	Oocyte retrieval	Oocyte retrieval
Size	<50 µm	80 to 300 µm (species dependent)	80 to 300 µm (species dependent)
Support cells	Few, very small	Numerous corona/cumulus	Numerous corona/cumulus
Nuclear status	Resting prophase I, Nuclear membrane	GV, has nuclear membrane	Resting MII, on temperature sensitive spindle, no nuclear membrane
Zona	No	Yes	Yes
Cortical granules	No	Central	Peripheral
Intracellular lipid	Little	May be abundant	May be abundant
Metabolic rate	Low	Low	Low
Surface: volume ratio	High	Low	Low

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Menezo and Guerin, this volume) in humans and farm species has led to a better theoretical understanding of the principles of cell freezing (Shaw et al, 2000; Pegg, 2002), and a significant improvement in cryoprotective agents (CPAs), and automated freezing equipment. These advances together with the increased survival rates of young oncology patients have generated a resurgence of interest in ovarian tissue cryopreservation.

Several options are currently available for primordial follicle banking, which include: (i) whole ovary cryopreservation (Revel et al, 2001; Wang et al, 2002) and (ii) ovarian cortex banking after oophorectomy or ovarian biopsy. If the latter option is selected, denuded primordial oocytes can be harvested from isolated primordial follicles and frozen, intact primordial follicles can be stored or alternatively and most commonly, primordial follicles can be frozen *in situ* within thin slices of the ovarian cortex (Gosden et al, 1994; Newton et al, 1996; Otkay et al, 1997). The development of protocols that optimize survival and developmental rates of primordial oocytes following exposure to the extreme chemical and physical stresses associated with cryopreservation has proven to be a major challenge. Reduced to its fundamentals, ovarian tissue cryopreservation requires that the mixture of cell types (stroma, follicle cells, and oocytes) found in the ovarian cortex tolerate the following non-physiological conditions: (i) exposure to molar concentrations of CPAs, (ii) cooling to subzero temperatures and (iii) removal or conversion of almost all liquid cell water into the solid state. Each of the different cell types may well have different cryopreservation optima. Furthermore, post-thaw survival is dependent on cooling and warming rates, as well as on the rate and method by which CPAs are removed from the cryopreserved tissue (Mazur, 1963; Pegg, 1987; Pegg and Karow, 1987).

CRYOPROTECTIVE AGENTS

The first and essential step in a cryopreservation protocol designed to support the recovery of viable primordial oocytes from ovarian cortex is to equilibrate the tissue segment in a highly water soluble, nontoxic and membrane permeable CPA. These agents penetrate the cell membranes and are thought to stabilize intracellular proteins, reduce the temperature at which cells undergo lethal intracellular ice formation and moderate the impact of concentrated intra- and extracellular electrolytes (Mazur, 1963). For gametes, embryos and ovarian tissues, the most commonly used CPAs are glycerol, propylene glycol, ethylene glycol and dimethylsulphoxide (DMSO). In the absence of CPAs, stromal cells, follicular cells and oocytes are first subjected to cold shock above the freezing point of the solution, and secondly, to a rise in osmolality before the solution freezes (Lovelock, 1953; Meryman, 1968) and may be irretrievably damaged by the formation of intracellular ice crystals (Mazur, 1963). The colligative properties of the CPAs therefore help protect all cells in the tissue

fragment against freezing injury by reducing the amount of ice crystal formation at low temperatures which in turn decreases external salt concentrations and reduces the likelihood of intracellular freezing. To avoid, or at least reduce the potential for freezing injury, the cooling rates for tissue slices should be fast enough to minimise exposure of cells to high intracellular concentrations of electrolytes and slow enough to avoid damaging intracellular ice formation. Finally, the thawing protocol used can profoundly affect oocyte and follicle survival rates. Post-thaw tissue survival can be improved by: (i) the slow removal of the cryoprotectant and (ii) by the inclusion of low concentrations of non permeable osmolytes, such as sucrose and mannitol which act as osmotic buffers against swelling during the addition and removal of the protective agents (Meryman, 1971; Mandelbaum et al, 1988). Significantly more pregnancies have been achieved in mice after orthotopic ovarian transplantation when fast (86%) rather than slow (25%) thaw protocols were used (Cox et al, 1996; Shaw et al, 2000).

One of the keys to the success of freezing complex ovarian tissues segments appears to be achieving adequate permeation of the tissue by the CPAs. High rates of solute penetration and high final concentrations of CPA must be achieved, and preferably at temperatures of about 4°C in order to minimize toxicity Newton et al, 1998). The problems of achieving adequate permeation of dense human ovarian cortex can be overcome by maximizing the surface area of the tissue for solute penetration (Gosden et al, 1994; Newton et al, 1998). This is most commonly achieved by preparation of thin strips of tissue that are 1-2 mm thick with a large surface area. Alternatively, the tissue can be dissociation of into follicles or isolated oocytes before freezing Cox et al, 1996). It is worth noting that where tissue is to be used to restore fertility by autografting, it is preferable to store tissue in strips rather than as small cubes or as isolated follicles or primordial oocytes, as this will facilitate the reattachment process.

COOLING TO SUB ZERO TEMPERATURES

When CPAs are frozen in molar concentrations, cell survival is strongly dependent on cooling rate, while the optimum cooling rate that yields maximum survival is dependent both on the type and concentration of CPA. Cell survival is equally dependent on warming rate. The optimum warming rate is dependent both on the CPA and its concentration, as well as on the preceding cooling rate. Cryobiological studies have shown that different types of cells, even when frozen in the same solution, exhibit different optimum rates. These facts are especially relevant to the cryopreservation of ovarian cortex, since this tissue comprises many diverse types of cells and each type has its own characteristic size, shape and permeability properties. Therefore, cooling and warming conditions

that are optimum for one cell type within the cortex may be damaging to other types of cell.

OSMOTIC EVENTS DURING CRYOPRESERVATION

When aqueous solutions of cells are frozen, free water is removed in the form of ice, causing solutions to become increasingly concentrated with decreasing temperature. The reverse occurs during thawing. The passage of water during slow (< -2°C/min) freezing typically dehydrates the tissue causing osmotic contraction (Lovelock, 1953; Mazur, 1963; Pegg and Karow, 1987) and the cells can suffer damage due to long exposure to high electrolyte concentrations, excessive cell dehydration, and the mechanical effects of external ice. When frozen cells are warmed rapidly, the melting of the cell suspension is equivalent to rapid dilution of the CPA that became concentrated during the freezing process. The rapid influx of water into cells or tissues as the extracellular milieu begins to melt can cause osmotic shock at subzero temperatures (Leibo, 1976). Sensitivity to osmotic shock is therefore a function of the cell's permeability to water and solutes. Yet another osmotic shock may occur during removal of the CPA after the cells are warmed and thawed. This osmotic shock can be prevented by use of any one of various mono- and disaccharides such as sucrose which act as osmotic buffers Leibo and Oda, 1993; McWilliams et al, 1995).

CURRENT PROTOCOLS USED FOR OVARIAN TISSUE FREEZING

Cryopreservation protocols for ovarian tissue can be broadly classified as “slow” or “rapid” according to the cooling rates and additives used. However the basic concepts of these protocols are the same as they both aim to protect the cells from the effects of chilling, intracellular ice crystal formation, dehydration and CPAs toxicity at both high and low temperatures. Because of the complexity of tissue architecture, ovarian freezing protocols must strike a balance between the optimal conditions required for each cell type. Although protocols are still far from optimized and despite the apparent difficulties of freezing complex tissues, the storage of ovarian tissue has nonetheless proved surprisingly successful as evidence by numerous reports demonstrating that human primordial follicles can survive cryopreservation after slow cooling to -196°C and storage in liquid nitrogen (Gosden et al, 1994; Hovatta et al, 1996; Newton et al, 1996; Oktay et al, 1997; Radford et al, 2001; Picton and Gosden, 2000; Oktay and Karlikaya, 2000).

SLOW FREEZING PROTOCOLS

For the most part, cryopreservation procedures used for the slow freezing of ovarian cortex are very similar to the procedures designed for the cryopreservation of cleavage stage embryos. The most efficient method for the preservation of human ovarian cortex involves equilibration of thin slices (1-2 mm thick) of ovarian cortex for 30 minutes at 4°C in excess freezing solution containing either 1.5M DMSO or ethylene glycol together with 0.1-0.5M sucrose (Netwon et al., 1996; Oktay and Karlikaya, 2000; Radford et al 2001). Equilibration of tissue with the CPA is aided by gentle rolling or shaking of the tissue slices. After transfer of the tissue pieces to labelled cryovials or ampoules, the tissue is loaded into the chamber of an automated freezing apparatus and the temperature is slowly lowered at a cooling rate of -0.3 to -0.5°C/min. The vials or ampoules are seeded to induce ice formation at -70°C, and cooled at -0.3°C/min to approximately -40°C, then at -10°C/min to -180°C, and finally plunged into liquid nitrogen for storage. No further major changes take place within the cytoplasm at temperatures below -130°C as this is the glass transition temperature of water. At -196°C ovarian tissues can be stored in liquid nitrogen indefinitely. The observation that embryos and spermatozoa do not deteriorate even after storage for decades in liquid nitrogen (Leibo et al, 1994; Fogarty et al, 2000) is particularly relevant to the storage of ovarian tissue for pediatric cases when it may be 20-30 years before the tissue will be required to restore fertility. When required, the cortical slice is thawed rapidly by swirling in a water bath at room temperature, and the CPA is progressively diluted from the tissue by serial 10-minute rinses in fresh medium at 4°C. With this approach, high post-thaw survival rates have been recorded for human primordial follicles of 84% and 74% for 1.5M ethylene glycol and DMSO, respectively, compared with survival rates of only 44% and 10% with 1.5M propylene glycol and glycerol, respectively (Newton et al, 1996). Similar low survival rates were previously recorded for glycerol by Green et al (1956) that was probably due to osmotic stress associated with this slowly permeating solute.

The exceptions to this general approach for the slow freezing of ovarian cortex are studies published by Harp et al (1994), Gunasena et al (1997) and Salle et al (1999). In the two former studies, samples were cooled at -0.5°C/min to approximately -55°C and then plunged into liquid nitrogen, and in the latter the samples were cooled at -2°C/min to -140°C prior to placement in liquid nitrogen.

RAPID FREEZING PROTOCOLS

An alternative to the slow freezing protocols detailed above is rapid freezing or vitrification. Here, high concentrations of CPA dehydrate the

cells before the ultra-rapid cooling rates allow the solutions to form glass (vitrify) rather than ice crystals (freeze) when they are plunged into liquid nitrogen (Rall, 1992; Pegg, 2002). For a number of species, the efficiency of vitrification has been improved for isolated cells such as oocytes and embryos by increasing both CPA concentration and the rate of cooling, while minimizing the cytotoxic effects of high CPA concentrations through the use of very small volumes of freezing solution with open-pulled straws Vajta et al (1998), cryoloops (Lane et al, 1999) or electron microscope grids (Martino et al, 1996). A major advantage of vitrification is that the expensive equipment used for slow freezing protocols is not required and the time required to freeze the cells is significantly decreased. However, ultra-rapid freezing regimens have proved less successful with human embryos (Trounson et al, 1988) and unpredictable with complex ovarian tissue slices. The cooling rates and storage vessels for large pieces of tissue need to be further optimized to prevent irreversible damage occurring if vitrification is to compete with slow freezing as a means to bank cortical slices. Furthermore, there is a question of long-term stability in a "glassy state" for vitrified cells as such samples may be prone to fracture damage and issues such as the most appropriate storage conditions (i.e. liquid or vapour phase) which also permit routine access to storage tanks need to be addressed.

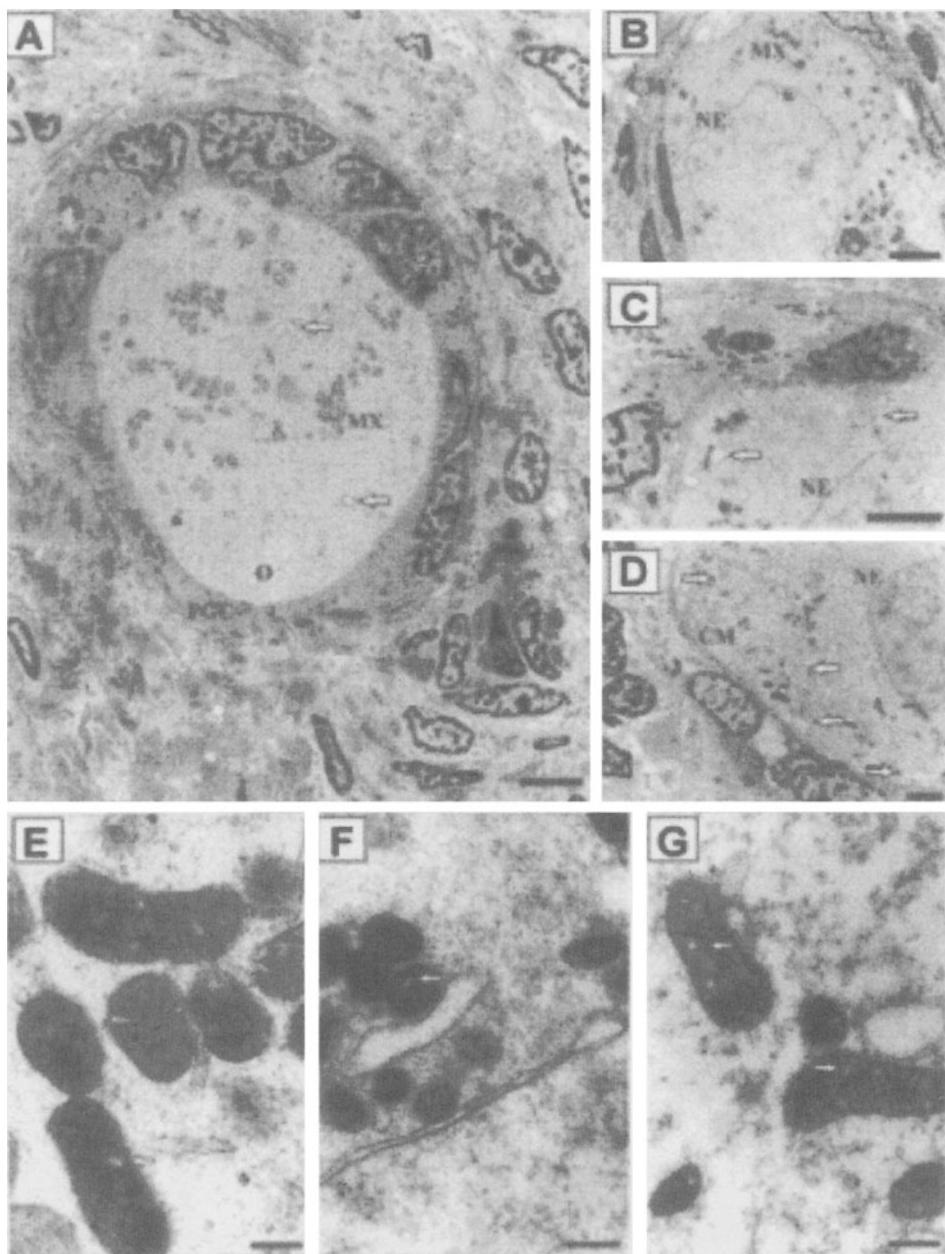
PRACTICAL ISSUES

There are a number of practical problems associated with the cryopreservation of ovarian tissue slices compared to isolated oocytes. Firstly, tissues respond very differently to ice formation compared to cell suspensions. Secondly, cells in tissues are usually closely packed, and they also have interacting connections with each other and with basement membranes. Fine capillaries or other blood vessels may also traverse tissues. Thirdly, tissues have three-dimensional structure that cell suspensions do not. Changes in both extracellular ice surrounding a piece of tissue, and recrystallization during warming of intracellular ice within cells of the tissue is therefore more likely to cause damage. Nonetheless, using the slow freezing approach it is possible to obtain high acute post-thaw follicle survival rates for human primordial follicles (Newton et al, 1996; Otkay et al, 2002) with normal tissue morphology at both the light (Newton et al, 1996; Salle et al, 1999) and electron microscopic levels (Gook et al, 1999). However, it is clear that the choice of an inappropriate CPA together with poor freezing, seeding and thawing practices can lead to extensive cellular damage which will compromise long-term tissue viability (Picton et al, 2000) (Figure 17.1). This damage may be partially explained by the fibrous and dense texture of human ovarian cortex which makes the tissue less permeable to CPA than ovarian tissues from other species. Although adequate CPA permeation of tissue fragments can be promoted by maximising the surface area (Cox et al,

1996;Hovatta et al, 1996;Newton et al, 1998;), it must be noted that until the methodology for the restoration of fertility using frozen-thawed tissue is demonstrated, the best option for the conservation of fertility through ovarian cortex freezing remains to freeze the primordial follicles *in situ* in strips of tissue, as this tissue is suitable for autografting, xenografting and *in vitro* growth. Finally there is the risk associated with viral transfer in liquid nitrogen storage tanks (Tedder et al, 1995).

The practice of ovarian cryopreservation has been widely and very rapidly adopted in many hospitals and private clinics as a means to preserve female fertility. Ideally these centers should be associated with reproductive medicine specialists and assisted conception units, but this is not always the case. From review of the published literature, it is clear that many different freezing practices have been adopted in these centers, often without paying sufficient attention to the experimental nature of the practice and/or the future application of the technology. This negligence may raise false expectations of the likely success of the procedure for vulnerable patients. There is current and a clear need to standardize ovarian freezing protocols, patient selection criteria, patient counselling, consents and record keeping if ovarian cryopreservation is to be offered as an experimental treatment. Additionally clinics should provide their ovarian freezing practitioners with suitable training which should include an audit of ovarian freezing practices before patient treatment is initiated. The latter can be achieved in the clinic setting through the use of post-thaw follicle harvesting and viability staining procedures for isolated follicles of either animal tissue or human ovarian tissue donated for research. Consequently, the consensus of many of those working in this rapidly expanding field is that it is preferable that ovarian cryopreservation should be conducted by specialist centers. These centers ideally will enjoy a close working association between adult and pediatric oncology services and reproductive medicine specialists and who have access to both tissue banking facilities and research laboratories for tissue screening for malignancy and where tissue functionality tests such as xenografting (Oktay et al, 1998) and/or tissue culture (Picton and Gosden, 2000) can be used to assess the long-term survival and post-thaw developmental potential of cryopreserved ovarian tissue.

While there is uncertainty amongst professionals on how best to preserve the fertility of young patients as ovarian cryobiology continues to develop, there are also specific psychological concerns about the extent to which children and adults should be involved in the decision-making process in general and discussions of which treatments are available in particular (Grundy et al, 2001). Furthermore, although the surgical



removal of ovarian tissue may be of high risk if taken in isolation, within the context of the patient's illness it may pose only minimal additional risk and in the long-term may actually benefit the individual concerned if fertility can be restored.

RESTORATION OF FERTILITY

Although the cryopreservation protocols for human primordial oocyte banking are far from optimized, they are nonetheless effective with respect to laboratory procedures, cost and storage. Experience suggests, however, that far greater technical problems will be encountered when the stored tissue is thawed and used to restore fertility. To date the options available for fertility restoration include autografting, xenografting or the complete *in vitro* growth of follicles and maturation of oocytes.

AUTOGRAFTING

The developmental potential of cryopreserved primordial follicles has been assayed *in vivo* by autografting (Carroll and Gosden, 1993) or xenografting of cryopreserved tissue under the kidney capsules of immunologically tolerant SCID mice (Oktay et al, 1998). In the human xenografted model, both stromal and primordial follicles survive the freeze-thaw and grafting procedures with follicle growth initiated and progressive up to early antral stages (10-12 layers of granulosa cells) after supplementation with recombinant human FSH (Oktay et al, 1998). These findings support observations in the marmoset monkey model where large (1-2 mm diameter), estrogenic antral follicles developed 21-32 days after

FIGURE 17.1. Transmission electron micrographs of primordial oocytes (O), pre-granulosa cells (PGC) and granulosa cells (GC) in human ovarian cortex before (a,b,e) and after cryopreservation (c,d,f,g). Tissue before freezing (a,b,e), showing an intact cell membrane (CM), nuclear envelope (NE), and mitochondria (MX). Vacuolation is indicated by the arrows. Morphologically normal tissue after cryopreservation (c) showing limited vacuolation, intact cell membrane and morphologically normal mitochondria. Tissue damaged during cryopreservation (d, f, g) showing intact cell and nuclear membranes but extensive vacuolation throughout the cytoplasm and in the mitochondria (arrows). Images (e-g) show high power magnification of mitochondria in healthy tissue before freezing (e) and after cryopreservation with low (f) or high (g) levels of vacuolation. (a-d) scale bar = 5 μ m; (e-g) scale bar = 0.5 μ m. (Adapted from Picton et al 2000⁵ with permission).

transplantation of cryopreserved tissue (Candy et al, 1995). While immensely useful for research, the main drawback of the xenograft model is that oocyte fertility cannot be tested *in vivo*. Nevertheless, the primordial follicles and the few growing follicles present in the grafts appeared to be cytologically normal and fertility can be assessed *in vitro*.

After cryopreservation, autografted ovarian tissue has been shown to restore normal reproductive function as demonstrated by the production of live offspring in mice (Carroll and Gosden, 1993) and sheep (Gosden et al, 1994). The restoration of fertility in the ovine is reassuring as the ovaries of these animals, although smaller than adult human ovaries, are bulkier, more fibrous and have a wider dispersal of primordial follicles than exists in murine ovaries. In the sheep experiments, tissue survival and developmental potential was assessed after freezing and thawing in 1.5M DMSO. Thin slices of frozen-thawed cortex were stitched back onto the ovarian pedicle and compared with unfrozen grafts attached to the contralateral side. Estrous activity was restored within 3-4 months in these animals and approximately nine months after grafting, eleven of the twelve grafts contained follicles. Although few follicles were present in these animals nine months after grafting, there were no obvious differences between frozen and fresh autografts at this stage, nor was there any evidence that either follicle dynamics or ovarian cycles were abnormal. Indeed, two of the six animals carried pregnancies to full term, one originating from the ovulation of a fresh graft and one from a frozen-thawed graft (Gosden et al, 1994). In a second group of animals, normal ovarian cyclicity was maintained for at least 22 months after ovarian cryopreservation and orthotopic autografting (Baird et al, 1999).

Clearly the ability of frozen-thawed ovarian tissue to re-form functional ovaries capable of folliculogenesis, steroidogenesis, ovulation and luteal function suggests that all major ovarian cell types, or their stem cells, were able to survive freezing and thawing. Follicles may, however, be lost at each stage in the procedure. The majority of these losses are not thought to be due to cryopreservation but rather depending on species (Gosden et al, 1994; Candy et al, 1995; Oktay et al, 1997; 1998), some 25-50% of the losses will occur as a result of grafting *per se*, which probably reflects the effects of acute ischemia, oxidative stress and reperfusion injury after grafting, as revascularization of the graft may take several days (Dissen et al, 1994). Improved revascularization through the use of exogenous antioxidants such as vitamin E (Nugent et al, 1998), together with the relative abundance of endogenous angiogenic growth factors (Koos, 1989), pituitary gonadotrophins and ovarian steroids (Sato et al, 1982; Dissen et al, 1994;) may help to reduce ischemia in the grafted tissue and so improve follicle survival.

On the basis of both animal experiments detailed above and recent clinical studies (Oktay and Karlikaya, 2000; Radford et al, 2001; Oktay et al, 2002), it is likely that the first successful cases of fertility restoration in humans using cryopreserved ovarian tissue will be achieved by

heterotopic or orthotopic autografting provided that uterine patency is maintained. Two recent reports of human autografting after cryopreservation (Oktay et al, 2000; Radford et al, 2001) have demonstrated that ovarian cortex survived the freeze-thaw-grafting process and that the tissue was capable of supporting limited folliculogenesis and steroidogenesis. The follicle losses during the freezing and thawing process and the grafting procedure were however unacceptably high. Unfortunately, none of these recent cases are suitable to judge the full potential of ovarian cryopreservation and autografting as an effective and clinically acceptable means of restoring fertility and therefore, this whole approach must be considered as experimental until the optimal site for grafting has been identified and a viable pregnancy has been achieved. While many questions concerning the validity of ovarian cryopreservation remain to be answered (Table 17.2), there is little doubt that the life span of the grafted tissue will be effected by follicle density, patient age at the time of cryopreservation, and prior exposure to chemo- or radiotherapies. Graft longevity will be further reduced by follicle losses associated with freeze-thaw injury and ischemia associated with the grafting procedure. Finally, the normality of uterine function and the contribution of the uterus to implantation and the establishment a viable pregnancy must not be overlooked during the restoration of fertility after successful cancer treatment.

TABLE 17.2. OUTSTANDING QUESTIONS RELATING TO THE DEVELOPMENT AND APPLICATION OF PRIMORDIAL OOCYTE BANKING AS A STRATEGY TO PRESERVE AND RESTORE FEMALE FERTILITY

Research	Application
<ul style="list-style-type: none"> • How long will ovarian grafts function? • How can follicle survival be improved during freezing and thawing? • How can follicle survival be improved after grafting? • What is the risk of returning malignant cell with the graft? • Will the oocytes produced after ovarian cryopreservation and autografting or <i>in vitro</i> growth and maturation be normal? • How long can tissue be stored 	<ul style="list-style-type: none"> • Is there an upper age limit? • How should this experimental technique be developed and regulated? • Which patient groups will benefit most? • Where should patients be treated? • How should practitioners be trained and audited? • Who should pay?

IN VITRO GROWTH AND MATURATION OF PRIMORDIAL OOCYTES

While the results of autografting experiments are encouraging, the most important proviso remains that the tissue stored for cancer patients does not harbour malignant cells that could reintroduce the cancer after grafting. Fortunately metastases do not always invade the ovaries (Oktay and Yih, 2002) and it seems unlikely that relapse would occur from ovarian grafts in patients with for example Wilm's tumour or Hodgkin's disease with low risk of ovarian disease (Kom et al, 2001;Oktay and Yih, 2002). However, no such assurances can be given for diseases such as leukemia where there is a clear need to quantify the relative risk of disease transmission. The data from Shaw et al. (1996) clearly demonstrates that ovarian tissue containing cancerous cells from blood borne disease has the potential to reintroduce the malignancy when the tissue is replaced. Although the risks of returning the old disease could in theory be minimized by screening the tissue with cytogenetic and molecular markers for the presence of malignant cells and by only transplanting tissue where there is a very low incidence of ovarian metastasis (Oktay and Yih, 2002), where any risk exists, an alternative strategy which will support the complete in vitro growth of oocytes from frozen-thawed primordial follicles should be used. Following fertilization by IVF or ICSI, this approach would theoretically enable embryos that are free of contamination to be transferred to the patient.

The development of cell culture systems that can support oocyte growth and maturation from the primordial stages will enable the potential of cortical tissue samples stored for the restoration of fertility to be maximized. Despite these possibilities, primordial follicles are the least understood of all stages of follicle development and the factor(s) responsible for the maintenance of the primordial pool or conversely, the activation and maintenance of primordial follicle growth, remain elusive (Picton, 2000). It is ironic therefore that the same characteristics that make primordial follicles suitable candidates for cryopreservation, make their culture extremely difficult (Table 17.1). It is, for example, relatively straightforward to harvest viable primordial follicles from cryopreserved cortex using a combination of enzymatic digestion and mechanical isolation techniques, but it has proved remarkably difficult to grow these isolated follicles in vitro over extended periods. The limited success achieved using mouse primordial (Eppig and O'Brien, 1996) and preantral (Newton et al, 1999) follicles have not yet translated to the culture of large animal or human primordial and preantral follicles. While this may be in part due to an inadequate understanding of primordial follicle biology (Picton, 2001), the length of the human follicular growth span from the primordial to Graafian stage (Gourgeon, 1996), stage-specific changes in the trophic requirements of the cells (Picton and Gosden, 2000), cellular

interactions, morphogenesis, and the sheer increase in bulk as the follicular antrum forms, all represent major challenges for human follicle culture technology. Consequently the relatively short culture protocols which have been used to produce live offspring in mice after the complete *in vitro* growth of primordial oocytes (Picton, 2001) have proved to be inappropriate for use with much larger human follicles which take several months to grow to maturity *in vivo*. The success of human follicle culture after cryopreservation is therefore likely to depend on the development of a new generation of culture technologies. A multi-step procedure which reflects the follicle growth dynamics *in vivo* is perhaps the most likely to succeed *in vitro* (Figure 17.2).

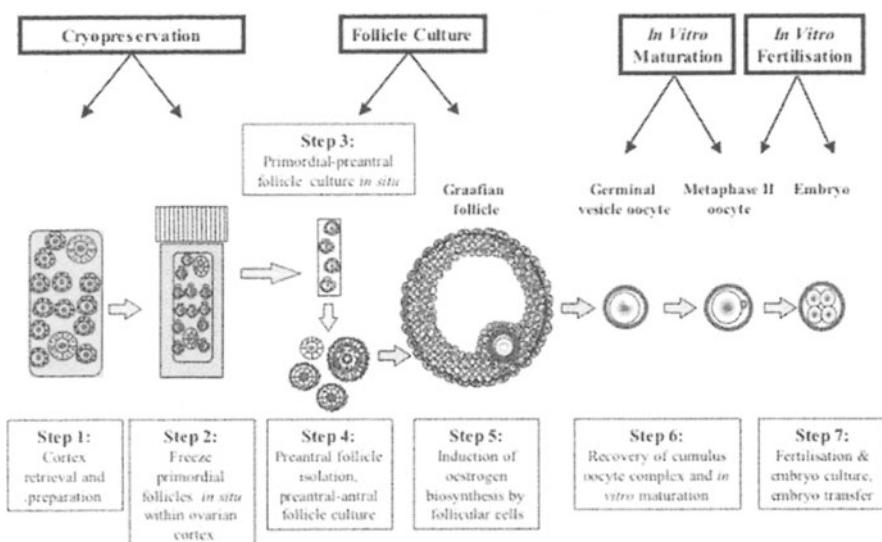


FIGURE 17.2. Steps involved in the complete *in vitro* production of metaphase II oocytes from primordial oocytes cryopreserved in ovarian cortex.

At present, the best strategy appears to be one that initiates follicle growth *in situ* followed by isolation of the follicles or granulosa-oocyte complexes once they have progressed to preantral stages. The final step requires the maturation of the corresponding oocytes with cumulus cell intact (Picton, 2002). The prospects of succeeding at each stage on order to produce a fertile gamete are likely to benefit by preserving cellular interactions and the phenotype of follicle cells as these provide the physiological environment in which oocytes normally develop *in vivo*. While progress has been made with the cryopreserved follicles of some animals (Eppig and O'Brien, 1996), progress with human tissue is slow and

is limited by the availability of tissue for research. A considerable effort is still needed to (i) develop follicle culture as a viable alternative to restoration of fertility by grafting; and (ii) to confirm that primordial follicle cryopreservation followed by extended culture does not induce epigenetic alterations in the imprinted genes in these *in vitro* derived mature gametes (Young et al, 2000).

RESEARCH PRIORITIES

Although the data reviewed in this chapter are encouraging, considerable basic research into ovarian cryopreservation and grafting, together with the continued development of follicle culture technology and improved drug treatment regimes are required in order to provide young patients with the hope of a safe and fertile future. There is a clear need to quantify both the likelihood of ovarian metastasis for the common childhood/youth cancers and the relative risk of ovarian failure induced by different classes of chemotherapy agents and radiation treatments (Arnon et al, 2001; Meirow and Nugent, 2001). Until such a time as healthy children are born after ovarian freezing and grafting or *in vitro* growth, the technology must be regarded as experimental. There are many practical and research issues to consider (Table 17.2) and intensive research effort should focus on the following:

- (1) Optimization of protocols for cortical tissue preservation through methodological improvements and through comparison of slow freeze and vitrification protocols.
- (2) Development of grafting strategies which promote rapid revascularisation and minimise ischemia in order to improve the efficiency of follicle survival and graft longevity
- (3) Development of highly sensitive tissue screening methods to detect the presence of malignant cells in order to quantify the risk of reintroduction of cancer through restoration of fertility by grafting.
- (4) Development of improved *in vitro* growth and maturation strategies for human follicles after ovarian tissue cryopreservation.
- (5) Investigation of the potential combination of xenografting for primordial to antral follicle growth with *in vitro* maturation of oocytes harvested from small antral follicles as a means to safely produce viable mature oocytes.
- (6) Investigation of alternative methods that can be used to protect the ovaries from the destructive effects of chemotherapeutic agents and radiation exposure.

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CHAPTER EIGHTEEN

CRYOPRESERVATION IN HUMAN ASSISTED REPRODUCTION

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INTRODUCTION

The use of superovulation techniques to improve and simplify human in vitro fertilization (IVF) leads to a problem of large numbers of oocytes and consequently embryos being produced. Solutions to avoid the creation of supernumerary embryos might include limiting the number of oocytes recovered or inseminated. Equally to avoid the risk of multiple pregnancies, it would be possible to discard supernumerary embryos, a notion that is usually unacceptable to both patients and clinicians. Fortunately, most IVF programs have addressed this issue with cryopreservation.

Mammalian embryos have been successfully cryopreserved and stored since 1972, when Whittingham and his colleagues obtained live mice after the transfer of cryopreserved morulae. Cryotechnology derived from rodents was applied commercially to cattle with more than 100,000 embryo transfers per annum being completed worldwide since 1980. In Australia, Trounson and Mohr obtained the first pregnancy from a cryopreserved embryo in humans in 1983. The application of cryopreservation techniques to human embryos spread rapidly and, with the help of optimized and simplified biological procedures, such as the use of propanediol as a cryoprotectant and the inclusion of sucrose in the cryopreservation solution (Lassale et al, 1985), this method became a routine adjunct in human IVF. In contrast to the situation in domestic species, human blastocyst cryopreserved was started later, in 1987, by Cohen and colleagues, but was then neglected until IVF blastocysts could be routinely obtained in greater numbers (Ménézo et al, 1992a,b).

While oocyte cryopreserved offers an alternative to embryo storage, little progress has been made in this field. The cryopreservation of ovarian tissue has gained interest, in particular for cancer patients whose medical treatment renders them susceptible to oocyte damage. These aspects are discussed below with respect to research issues as clinical application has been limited (see Picton, this volume).

GENERAL ASPECTS OF EMBRYO CRYOPRESERVATION

Cryopreservation of living cells implies cooling and freezing cells in order to put their life on "stand by." During this process, lethal damage can occur by lysis and impaired viability can result from intracellular ice crystal formation that can cause mechanical disruption of the cytoplasm or its components. The application of osmotic forces associated with dehydration results in increased internal concentrations of salts that can adversely affect viability owing to the removal of internal water that can induce damage by a process termed 'salting out.' Certain cryoprotectants such as ethylene glycol (Klug et al, 2001) may be toxic or even teratogenic. Thus extreme care needs to be taken in the selection of cryoprotectants and the method of dehydration and cooling to liquid nitrogen temperatures in order to reduce the potential for lethal or sublethal ice crystal formation. The selection of protocol is specific for different each cell type, including human embryos.

The fall in temperature during cryopreservation has a direct effect on the integrated biochemistry of the cell. For each 10°C decrease in temperature, a 50% decrease in biochemical activity is observed (Arrhenius relationship, Fig. 18.1). For example, a particular cell activity, such as one involving an enzymatic reaction that may require 1 second to complete at 27° C may require 2 million years at liquid nitrogen temperatures. For cell function, this temperature related phenomenon primarily effects levels of cellular metabolism and protein function owing to physical alterations in the gel like cytoplasm that is surrounded by a plasma membrane, which in turn is composed of bioactive proteins (e.g., receptors and transporters) embedded in a lipid bilayer. However, not all cellular activities decline in concert during cooling, which can result in a differential disturbances of metabolic processes and problems effecting cell viability may arise as a consequence during the freezing and thawing stages.

For the embryo, cryopreserved protocols are designed to cool at rates slow enough to remove all freezable water. However, it is important to understand that some proportion of intracellular water remains bound to molecules and structures and is not eliminated during dehydration and therefore, can remain in an unfrozen state. As the temperature continues to decrease, aqueous solutions surrounding cells undergo physical and chemical changes such that

membranes can be adversely affected by osmotic shock as the hypertonicity of external solution increases. The increase in external salt concentrations within the residual liquid compartment results in water being withdrawn by osmosis from the cell as a consequence of passive dehydration and shrinkage. Excessive or uncontrolled reductions in cell volume can lead to irreversible cytoskeletal damage and cell death.

Temperature	27°C	4°C	-73°C	-173°C
Duration Time	1 sec	4 sec	50 min	$2 \cdot 10^6$ yrs

FIGURE 18.1 Relationship between temperature and the time required for a theoretical reaction to occur in a cell.

Another major problem associated with cryopreservation is ice crystalization. As temperature declines, the proteinaceous gel that constitutes the cytoplasm is normally less likely to freeze than the extracellular compartment. But if freezable water remains in the cell, it will form ice crystals that can grow and lengthen as temperature falls that will cause major lesions in the plasma membrane and in other membranous elements. Slow cooling is intended to avoid cytoplasmic ice formation and for early human embryos, a cooling rate of -0.3°C per minute seems optimal as the cryoprotective solutions with which the embryo is pre-equilibrated usually reach temperatures as low as -15°C before ice is formed. This phenomenon is known as super-cooling and releases latent heat that is seen on cooling curves as a short break in the programmed linear decrease in temperature. The avoidance of supercooling is essential for the success of embryo cryopreservation, and is usually prevented by inducing ice formation at about -7°C . This process is commonly termed 'seeding' and involves brief contact with the surface of the cryoampoule or straw with forceps or other probes previously cooled in liquid nitrogen. When embryos are cryopreserved in straws, seeding is performed on small air-filled spaces on either side of a column of fluid in which the embryos are contained.

Cryopreservation by vitrification (i.e., glassification) works on a different principle and was one of the first methods of liquid nitrogen storage applied to cells. Because the formation of internal or external ice may be detrimental for cell survival, cryopreservation in the absence of ice formation involves

equilibrating cells in very high concentrations of cryoprotectants. A vitreous (i.e., glass) state is obtained by ultrarapid cooling that precludes crystallization. For embryos, straws or cryoampules are transferred directly from room temperature to liquid nitrogen (-196°C). This method has the potential to avoid most of the lethal effects of unintended ice formation and as a result, the absence of salting out can also avoid toxic "solute effects." The vitrification process usually involves a mixture of DMSO, acetamide, propanediol and polyethylene glycol at very high molar concentrations. However, toxicity of these solutions is the principal drawback to the use of this method for human embryos with potential effects on chromosomal structure of particular concern. There are also intrinsic problems related to the thermal exchange between vessel and liquid nitrogen, with the actual rate of drop limited by the conduction of the embryo-containing vessel, which if inappropriate, may affect viability. Based on the simplicity of the technique and its longstanding availability in animal systems, one might have expected that vitrification would have replaced classical slow cryopreservation protocols in current clinical use. This has not been the case, which probably is related to the absence of a demonstrable effect on the frequency of congenital anomalies in IVF babies from thawed embryos cryopreserved by slow cooling methods. A summary of cryopreservation protocols for mammalian embryos is shown in diagrammatic form in figure 18.2.

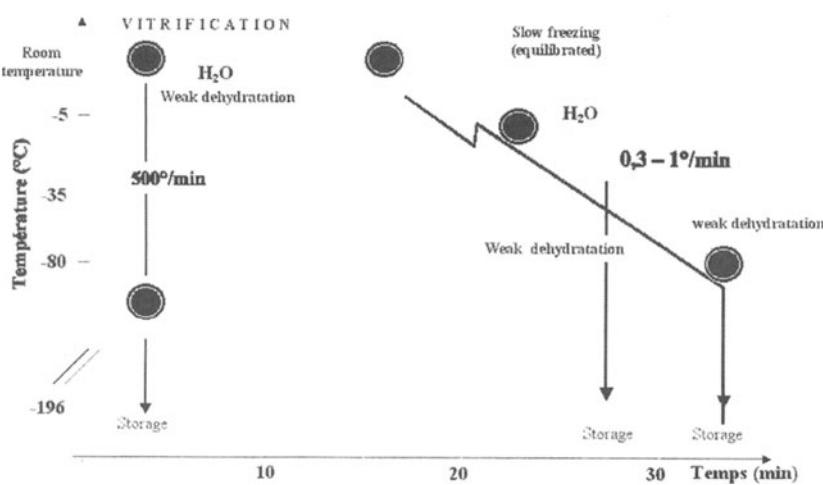


FIGURE 18.2. Slow cooling and vitrification are two methods currently used to cryopreserve early mammalian embryos, including the human.

CRYOPROTECTANTS

The first use of a cryoprotectant in reproductive biology was reported by Polge and colleagues (1949) who used glycerol to cryopreserve sperm. There are two general classes of cryoprotectants, those that are cell permeable and those that are impermeable.

CELL PERMEABLE CRYOPROTECTANTS

As these reagents enter cells and partly displace water, they tend to maintain cell volume and to some extent intracellular architecture, while reducing the formation of the 'ice front' if crystallization occurs inadvertently. Dimethyl sulfoxide (DMSO), glycerol and 1,2- propanediol are cell permeable agents commonly used in slow cooling human embryo cryopreservation. Acetamide has been used for vitrification, but these and others used in this method may be toxic. While ethylene glycol has been used in animal systems, it is not appropriate for the human. The use of glycols as cryoprotectants is always questionable because these can often be cytotoxic (Van der Elst et al, 1995; Demirci et al, 2001,) or if metabolized, their derivatives may also be cytotoxic (Klug et al, 2001). While glycerol is easily metabolized by all types of cells, it appears to be toxic for the oocyte, perhaps as a result of suboptimal rates of penetration. These agents are usually viscous, water soluble and used at high concentration. The rate of penetration is temperature dependent and will differ according to the specific chemical structure and cell type, including mammalian embryos

NONPERMEABLE CRYOPROTECTANTS

These reagents represent a wide spectrum of compounds from derivatives of starch (hydroxyethyl starch), polyethylene glycol and polymers such as polyvinlypyrrolidone (PVP), to proteins and low molecular weight sugars such as sucrose, trehalose, lactose, galactose and mannitol. The addition of one or more of these compounds to the cryopreservation solutions is thought to limit crystallization and control osmotic shock.

CRYOPRESERVATION IN STRAWS OR AMPOULES ?

Embryos can be cryopreserved in glass ampoules, plastic vials or straws. However, in our experience, *straws* are preferable because they are generally easier to handle, have a higher thermal conduction than glass, and may be safer, as suggested by reports of glass ampoule explosion during thawing. However, container selection is usually program-specific and based on outcome experience.

COMPOSITION OF CRYOPRESERVATION SOLUTIONS

Supplementation with serum or albumin in both freezing and thawing solutions containing both phosphate and bicarbonate are customary for human embryos. Our experience indicates that phosphate buffered solutions are preferable for cleavage stage embryos while bicarbonate buffered solutions, such as embryo culture media, may be preferable for the blastocyst stages. While phosphate buffered solutions require no equilibration CO₂ to maintain a constant pH, equilibration is mandatory, even at room temperature, for the bicarbonate buffered media. Protein supplementation in current use can originate from human or animal sources including human fetal cord serum, autologous or pooled maternal samples, fetal calf serum, or commercially prepared human serum albumin (HSA). It has been suggested that a possibility exists for viral transmission with the use of serum, especially if pooled maternal or umbilical cord sources are used, and it may be wise for programs to institute systematic pathogen screening of their source material. While supplementation with HSA is common, Warnes et al (1997) suggested it is less efficient than whole serum as an additive in cryopreservation solutions. So-called 'serum substitutes' have also been used but there are no studies on the comparative efficiency of these compounds and for some commercial preparations, the actual biochemical composition of the substitute is undisclosed.

CURRENT PROTOCOLS FOR HUMAN EMBRYO CRYOPRESERVATION

GENERAL PRINCIPLES

Human embryos have been successfully cryopreserved by slow cooling protocols in solutions containing 1,2-Propanediol (PROH; (Lassale et al, 1985), DMSO (Trounson and Mohr, 1983), or glycerol (Ménézo et al, 1992). For each cryoprotectant, optimal efficacy is stage-specific and related to its ability to penetrate blastomeres. For thawing, the stepwise removal of the cryoprotectants is mandatory as this progressive rehydration reduces abrupt changes in cell volume (Schneider and Mazur, 1986). For early cleavage stages, rehydration usually occurs in two steps while for the blastocyst, two or 2 or more steps are required. For vitrification the opposite applies, namely, the faster freezing is accomplished the more rapid is the rehydration, which is usually accomplished in a single step.

The careful storage of embryos is an essential, albeit often overlooked element of a successful ART cryopreservation program. Tyler et al (1996) stressed the importance of a rapid handling of vials and straws that contain

cryopreserved embryos as daily opening and closing of the liquid nitrogen dewars associated with removal or addition of samples could create temperature differentials which are difficult to manage and whose effect, if any, on other stored embryos is unknown. There is no impairment of embryo viability after at least 8 years of carefully managed storage (Wang et al, 1994), but for practical purposes, we suggest a maximum of 5 years.

STAGE-SPECIFIC PROTOCOLS

PRONUCLEAR AND CLEAVAGE STAGE EMBRYOS

The current and common method for the cryopreservation of human pronuclear (PN) and early cleaving stage embryos uses PROH and sucrose as first described by LaSalle et al (1985) and Testart et al (1986), which involved methods that originated from animal studies by work of Renard et al (1984). This protocol has been remarkably successful when embryos are slow-cooled according to the following program: the first 'ramp' down to a seeding point of -7°C has a slope of -1 to -2°C per minute, followed by seeding and additional cooling at $-0.3^{\circ}\text{C}/\text{mn}$ to -30°C . Generally, a hold time of about 90 sec is introduced prior to seeding followed by a second hold of 5 min after seeding. Cryovessels are plunged directly into liquid nitrogen, usually after a few minute hold at -30°C . During thawing, cryoprotectant removal is achieved in a stepwise fashion and in optimal survival rates are only obtained when sucrose is combined with PROH. Propanediol alone leads to reduced survival rates (32 % vs. 62 %) with only 10 % of embryos intact after thawing, as compared to 44 % with PROH and sucrose (Mandelbaum et al, 1988). The DMSO cryopreservation protocol introduced by Trounson and Mohr (1983) has the disadvantage of being longer than the PROH method and provides no apparent benefit with respect to outcome, despite claims to the contrary (Van der Elst et al, 1995), and as a result the latter protocol is currently the procedure of choice for pronuclear and early cleavage stage embryos. Typical outcome findings reported for pronuclear and cleavage stage embryos from several programs are summarized in Tables 18.1 and 18.2, respectively.

El Danasouri et al (2001) recently advocated the use of vitrification for cleavage stage embryos on day 3. However, the reported survival rate seems low and perhaps best suited for intact and normal appearing 8-cell embryos. Currently, this is no clear evidence that vitrification improves outcome and the aforementioned concerns of reagent toxicity remain.

CRYOPRESERVATION AT THE PRONUCLEAR OR CLEAVAGE STAGES?

Cryopreservation at the pronuclear stage has the potential advantage of avoiding significant chromosomal disruption because there is no meiotic spindle and chromatin is enclosed by a membrane. Scott and Smith (1998) and Tesarik and Greco (1999) have described criteria for determining the viability of pronuclear stage human embryos that include pronuclear position, number and the distribution of nucleoli and certain cytoplasm characteristics (see Scott, this volume), and in this regard, it has been suggested that cryopreservation should be performed when the juxtaposed pronuclei are centrally located. Post-thaw survival rates for pronuclear embryos have been reported to be at least >60% (56-93%) with pregnancy rates/transfer in the 17-to-30% range (Al Hasani, 1996; Hoover, 1997).

The evaluation of cleavage stage human embryos in order to select those with the highest potential to survive thawing is probably easier to perform at the pronuclear stage because while most pronuclear embryos appear similar, the same cannot be said for the cleavage stages. For purposes of cryopreservation during the cleavage stages, appropriate cell number and the degree of fragmentation, if any, need to be considered. Antczak and Van Blerkom (1999) have recently pointed out the importance of fragmentation patterns in early human development with both extent and distribution major features. The presence of multinuclear blastomeres must also be determined as such embryos have reduced potential owing to chromosomal anomalies (Staessen and Van Steirteghem, 1998). As a guide, an appropriate embryo for cryopreservation during cleavage has four blastomeres on day 2, 6-to-8 cells on day 3, no multinucleate blastomeres and a degree of fragmentation below 15% (see Van Blerkom, this volume). With these embryos, survival rates after thawing are usually in excess of 60% (11-93%) with pregnancy rates in the 20% range. The implantation potential of cryopreserved cleavage stage embryos is directly related to the number of blastomeres that survive intact (Edgar et al, 2000). With respect to whether cryopreservation should be performed at the pronuclear or early cleavage stages, our experience supports the contention of Kattera et al (1999) that this process is optimized with cleavage stage embryos exhibiting the above characteristics, with 8-cell embryos having the highest probability of producing a pregnancy. These findings also apply to vitrification.

ICSI has a negative impact on the success of cryopreservation for cleavage-stage human embryos. Van den Abbeel et al (2000a) reported a 3% implantation rate per thawed embryo. It has also been reported that vitrification is more deleterious for ICSI embryos than the PROH method (Mauri et al, 2001), with embryonic losses as high as 57% after vitrification.

It seems likely that incomplete healing of the oocyte cytoplasmic membrane and or zona pellucida after ICSI may lead to defects that persist at the time of cryopreservation, which may be linked to high frequencies of zona pellucida cracking and reduced blastomere survival in such embryos. This notion is supported by outcome results after thawing of biopsied 8-cell embryos in which passage through the zona pellucida is made to retrieve a blastomere (Van den Abbeel and Van Steirteghem, 2000b). In this respect, outcome with ICSI embryos cryopreserved at the pronuclear, cleavage and blastocyst stages

TABLE 18.1 OUTCOME RESULTS FROM CRYOPRESERVATION AT THE PRONUCLEAR STAGES

	*1	**1	***2	*3	**3
Thawed	744	333	1005	197	504
E Recovered	569	261	790	127	325
% Recovery	76.5	78	79	64	64
Clin PR/Tr (%)	17	20	19	14.8	14.6
%Implantation/ E.tr	6	7	10.4	3.8	
Take home/100E. tr	?	?	9.3	3.8	5.2
Take home/100 Fr E.	?	?	7.4	2	2.8

*ICSI, **IVF, ***mixed

1- Al Hasani et al. 2- Senn et al (24hrs culture before transfer) 3- Kattera et al (24 hrs culture before transfer)

may indicate whether extended culture (i.e., to the morula or blastocyst) promotes healing and the efficacy of the cryopreservation process. In any event, it seems prudent to thaw embryos a few hours or even a full day before transfer, which would enable a meaningful observation of those dynamic parameters associated with competence, such as the resumption of mitosis. It has been reported that extended culture prior to transfer doubles the "take home baby rate" from 2.7 to 6.5% (Van der Elst et al, 1997), and a 24 hour culture may be especially relevant for transfers involving pronuclear embryos (Kattera et al, 1999; Senn et al, 2000). At present, however, whether cryopreservation is best performed at the pronuclear or cleavage stages remains unresolved.

BLASTOCYST CRYOPRESERVATION

There appears to be some significant advantages to the cryopreservation of human blastocysts (see Veiga, this volume). Blastocyst development in co-culture or sequential media systems prior to cryopreservation clearly is associated with increased cell numbers and may permit the selection of high competence embryos (Janny and Ménézo, 1994,1996). Theoretically, blastocysts are easier to freeze for two reasons: first, the nucleo-cytoplasmic ratio is higher and second, the higher cell number may allow recovery with retained viability if some proportion of cells are destroyed after thawing. Glycerol has been the cryoprotectant of choice for blastocysts in animal systems, although ethylene glycol is currently used for bovine and ovine embryos. We have developed protocols for human blastocyst cryopreservation using *in vitro* produced bovine embryos. However, with respect to outcome it is clear from other studies that important differences exist between *in vivo* and *in vitro* produced embryos (Leibo and Loskutoff, 1993). In our experience for example, glycerol seems to be a reasonable compromise as a cryoprotectant for the blastocyst. Glycerol was used in the first human blastocyst cryopreservation report by Cohen et al (1985) and as a first approach in our studies, the protocol was modified according to findings from the bovine, and subsequently simplified for clinical use (Ménézo et al, 1992; Ménézo and Veiga, 1997).

**TABLE 18.2 OUTCOME RESULTS WITH CRYOPRESERVATION
AT THE CLEAVAGE STAGES**

	1	2	*3	**3
E Thawed	3807	600	223	219
E Recovered	1871	442	165	164
% Recovery	49.1	73.7	74	75
Clin. PR/Tr (%)	9.4	10.9	19	16
%Implantation/ E.tr	4.7	5.9		
Take home/100E. tr	3.2	4.3	6.4	4.4
Take home/100 Fr E.	1.4	3.2	4.5	3.2

1- Van der Elst et al. 2- Senn et al. 3- Kattera et al *IVF,**ICSI

Typically, the addition of glycerol occurs in two steps of 10min duration with the addition of sucrose in the final step, which immediately precedes the start of the cryopreservation program. Likewise, thawing is simplified to require only two steps. The final protocol we developed uses a programmed cooling rate of -2°C/minute from 22°C to - 6.5°C, followed by manual

seeding after a 60 second hold. After seeding and a second hold of 3 minutes, cooling at a rate of - 0.3°C per minute from -6.5°C to -37°C is performed, followed by a direct plunged into liquid nitrogen. In all steps of embryo manipulation prior to actual cooling, blastocysts are maintained at room temperature under a stream of gas (5% O₂, 5% CO₂ and 90% N₂) owing to the use of bicarbonate buffered media in all solutions, including those used for thawing and rehydration. Thawed embryos are initially allowed to recover for 3-to-4 hours in the normal culture medium prior to transfer. However, with the advent of sequential media, embryos are currently cultured overnight. Only morphologically normal embryos (see Veiga, this volume) that have re-expanded are replaced in synchronized patients that had been prepared for transfer in either a natural cycles or one managed by hormone replacement. The blastocyst recovery rate is somewhat lower with a simplified thawing protocol, as compared to the multi-step method of Cohen et al (1985), but outcome results are comparable.

Our experience with blastocyst cryopreservation from 1995 to 1998 used embryos developed in a co-culture system and of the 100 blastocysts thawed, 10 babies were born, 20% of patients miscarried, and the sex ratio was; 53% males and 47% females (Ménézo et al, 1999). The highest implantation rates occurred in cycles with hormone replacement therapy (26.2%), which was not unexpected as transfers in natural cycles are associated with lower implantation rates (12 to 16%: Kaufmann et al, 1995). In our program, the use of sequential media for blastocyst culture was initially associated with an unexpected reduction in outcome, resulting in a re-evaluation of protocol such that at thawing, a slower rate of cryoprotectant removal was adopted (see Ménézo et al, 1992), resulting in term of pregnancy rates comparable to those reported by others (Tucker, 2001, Langley et al, 2001). The two different methods of rehydration are shown in figure 18.3. Outcome results from blastocyst cryopreservation reported by several programs are presented in Table 18.3.

VITRIFICATION OF BLASTOCYSTS

Vitrification using 'cryoloops' has applied to human blastocysts in a small number of IVF procedures with some success (Mikado et al, 2001; van der Zwalm et al, 2002). However, outcome reports are not yet a level sufficient to recommend vitrification of human embryos at any stage of preimplantation development, and there is some concern regarding the use of 40% ethylene glycol during stages of development where there is high mitotic activity. At present, its use does not seem to enhance outcome when compared to PROH or glycerol (Langley et al, 2001, Tucker, 2001). For example, catabolism of

ethylene glycol leads to the transitory formation of toxic aldehydes, which presents a potential risk that is difficult to justify.

TABLE 18.3. OUTCOME RESULTS WITH BLASTOCYST CRYOPRESERVATION USING SEQUENTIAL CULTURE MEDIA SYSTEMS

	Tucker	Langley	Behr	V D Z*
Thaw	58		64	39
Transfers	54	72		35
Viable Pregnancies	18	24	17**	5
% per thaw	31			13
% per transfer	33	33	26	15
Impl./ E Trfd (%)		21.9	16	18
Take home/Frozen E(%)		10.9		8

*Van der Zwalm et al. Vitrification with 40% Ethylene Glycol, 18% Ficoll, 0.3M Sucrose **DELIVERIES

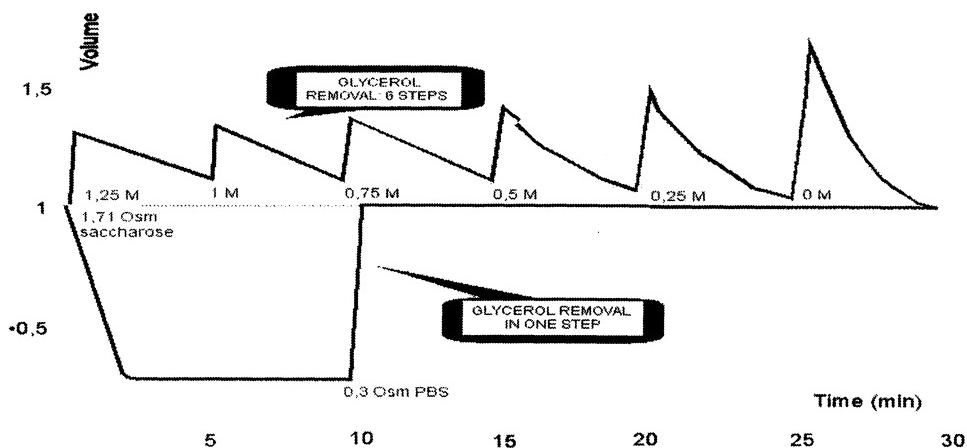


FIGURE 18.3. Protocol for rehydration for human blastocysts

IS ASSISTED HATCHING BENEFICIAL FOR CRYOPRESERVED EMBRYOS?

The rationale for assisted hatching in thawed embryos is that the freeze/thaw process might alter the physical structure of the zona pellucida and if it is sufficiently ‘hardened,’ render it less susceptible to lysis at the time of hatching after uterine transfer. It has therefore been suggested that microdissection of the zona might improve the outcome (Tucker et al, 1991). This, however, has not been confirmed by the observations of Matson et al, (1997) on mouse embryos and remains controversial for the human (see Wright and Jones, this volume).

QUALITY CONTROL AND ASSURANCE IN EMBRYO CRYOPRESERVATION PROGRAMS

In order to determine the quality and the efficiency of a cryopreservation program, it is essential that the following data be recorded and evaluated: (a) the cryopreservation rate of the extra embryos, (b) the cryopreservation rate per couple, (c) the recovery rate per cryopreserved/thawed embryo, and (d) the ‘take home baby’ rate per cryopreservation and per thawed transferred embryo. Unfortunately, this data is not usually presented in most outcome reports. In order to develop a universal method of assessing the relative and comparative success of cryopreservation programs, the following parameters are recommended:

- Fre:** Cryopreserved rate per excess embryo (not transferred)
- Frp:** Cryopreserved rate per couple, based on each 100 couples IVF program and the number of supernumerary embryos cryopreserved.
- FE:** Cryopreservation efficiency, $FE = Fre \times BTH$, where BTH is the ‘take home baby rate’ per cryopreserved embryo.

It is clear that when a center is highly selective in cryopreservation and few embryos are cryopreserved, Fre, Frp and FE may be low, while the apparent BTH may appear high. These parameters are rarely presented in the literature, but it can be assumed that for individual IVF centers their cryopreservation program reflects the IVF program in general, such that the mean number of oocytes retrieved, age of the patients and the policy for embryo transfer have real impacts on FrP. In our center for example, a mean number of 9-10 oocytes is retrieved, the fertilization rate is 65% in IVF and ICSI, and there is a mean transfer rate of 1.9 embryos per patient. The Fre is approximately 32%, and the Frp is approximately 31% for blastocysts. For patients with extra embryos, a mean number of 3 blastocysts was cryopreserved. For the French registry (FIVNAT), the Frp is around 20% with

most cryopreservation involving cleavage stage embryos. If we consider a BTH consistently below 10%, it can be concluded that the efficacy of blastocyst cryopreservation is low, perhaps because of the reduced number of embryos that develop to this stage.

IS THERE AN OPTIMAL STAGE FOR HUMAN EMBRYO CRYOPRESERVATION?

An analysis of published outcome reports for thawed pronuclear embryos indicates that on average, the birth rate per transferred embryo ranges from approximately 3-to-7% (El-Hasani et al, 1996; Hoover et al, 1997; Nikolettos et al, 2000; Senn et al, 2000), with similar results reported for early cleavage stages (3- to- 6 %; Van den Abbeel et al, 2000; Van der Elst et al, 1997; Kattera et al, 1999; Senn et al, 2000; Langley et al, 2000). For cleavage stage embryos, arrested development after thawing may result form biochemical or cytogenetic (Iwarson et al, 1999) defects that are not cryopreservation related, but rather represent intrinsic paternal or maternal defects (Janny and Ménézo, 1994, 1996; Delhanty, 2001) that would adversely effect development *in vivo*. The ongoing implantation rate of fresh cleavage stage embryos is around 12.5%, of which half would be expected to develop to the blastocyst under current culture systems. Embryo selection for competence during the cleavage stages is not an exact science and present morphological criteria for day 2 or 3 evaluations may be poor indicators of quality and potential (Graham et al, 2000, Milki et al, 2001; Scott and Van Blerkom, this volume). Recent progress in blastocyst culture has resulted in an increased interest in *cryopreservation* at this stage. The best results from thawed blastocysts currently provide a BTH of around 10% per embryo (Ménézo and Veiga, 1997; Tucker, 2001; Langley et al, 2000). In 1999, our outcome experience resulted in 39 babies from 346 thawed embryos of which 278 were transferred. However, outcome results with blastocysts are highly variable between IVF programs and lower results may occur in programs in which a high proportion of cycles involve ICSI. Although relatively limited to date, outcome experience with ICSI blastocysts indicates that they may be more susceptible to lethal cryodamage. While these findings tend to suggest benefits of embryo cryopreservation at the blastocyst stage, there is one advantage to cryopreservation at early stages that is often not considered, namely, the possibility of re-cryopreservation at a later stage if development in culture continues to the blastocyst stage.

Similar to most programs experienced in extended embryo culture, we prefer to freeze embryos at the blastocyst stage (Langley et al, 2001; Tucker, 2001; Behr, 2002) and to thaw up to 15 hours in advance of transfer. In general, outcome with thawed blastocysts is at least equivalent to the

pronuclear and cleavage stages, even accounting for the developmental arrest experienced by some proportion of early stage embryos. One obvious benefit of blastocyst cryostorage is that while fewer embryos are cryopreserved, those in storage are likely to have higher competence and thus fewer thawed embryos may need to be transferred, which could translated into a significant reduction in the frequency of higher order gestations (Ménézo and Veiga, 1997; Langley et al, 2001; Gerris, this volume). With careful selection for cryopreservation during the cleavage and blastocysts stages, implantation rates per embryo have been reported to be at least twice those described for cleaving stage embryos (Langley et al, 2001). However, it seems apparent that a general lack of expertise in blastocyst technology is one problem common in the IVF field, which may be largely due to the absence of a standard or optimal protocol. At present, different methods of culture, types of media, serum supplementation, osmolalities and gas phases are used and each may have a differential impact on the efficiency of blastocyst viability after thawing. As a general principal, Van der Elst et al (1997) and others suggest that transfer of pronuclear or cleavage stage embryos only involve those with demonstrable evidence of at least one cycle of cell division during culture. For the blastocyst, full re-expansion prior to transfer seems to be an important empirical indication of viability, and as such, it seems reasonable to extend culture after thawing, especially when several embryos have been thawed simultaneously.

The implantation process is not well understood and as a result, there may be a significant amount of embryo wastage owing to improper preparation of the endometrium for fresh and thawed embryos (see Seppala and Lessey, this volume). One calculation of wastage suggests that as many as two thirds of transferred embryos may be lost owing placement in a uterine environment that is inconsistent with implantation (Kaufmann et al, 1995; Hoover et al, 1997; Ménézo and Veiga, 1997). When it is considered that most experienced IVF programs report successful outcomes in excess of 40% of initiated cycles, whether a hostile uterine environment can be responsible for such a high proportion of loss needs to be confirmed. However, the importance of a receptive endometrium cannot be overlooked as replacement into one that is ill prepared renders moot all previous efforts to produce and select competent embryos. Consequently, efforts directed to the identification of reliable markers of uterine receptivity are critical if the full potential of IVF with fresh and thawed embryos is to be achieved.

OOCYTE AND OVARIAN CRYOPRESERVATION

In some instances of infertility treatment, it may be useful to cryopreserve oocytes rather than pronuclear or later stage embryos. While numerous oocytes have been thawed and fertilized by ICSI, to date only a few babies have been born and as a result, this approach in its present form does not appear to be a viable option in infertility management. As in animal studies, the stage of meiotic maturation best suited for cryopreservation, germinal vesicle (GV) or metaphase II (MII), is unresolved for clinical use. One of the most significant issues with oocyte cryopreservation is the temperature sensitivity of the metaphase spindle and as a result, it may be more appropriate to freeze at the GV stage where DNA occurs as chromatin enclosed by a nuclear membrane. However, the GV stage is problematic as in vitro maturation (IVM) after thawing is required to achieve normal fertilization competence and while current IVM systems may promote nuclear maturation to MII, embryos resulting from ICSI fertilization are largely developmentally incompetent (see Cavilla and Hartshorne, this volume). However, there have been some potentially useful results from this area of clinical research including the finding that the presence of intact cumulus cells is not mandatory for oocyte cryopreservation using the PROH slow-cool/rapid thaw process and that increasing the sucrose concentration to 0.3 M improves outcome (Fabbri et al, 2001). The option of MII oocyte cryopreservation could be proposed when a semen sample cannot be produced at the time of follicular aspiration. With current protocols for MII oocytes, a pregnancy rate per attempt $\leq 10\%$ may be anticipated (Fabbri et al, 2001).

While vitrification is another method for oocyte cryopreservation, the few clinical outcome reports show very poor success rates (Kuleshova et al, 1999; Wu et al, 2001). The issue of toxicity associated with high cryoprotectant concentration may increase the risk of aneuploid embryos and fetal malformations (Kola et al, 1988), and as such should be a matter of serious concern. At present, oocyte cryopreservation should only be contemplated in unusual or 'desperate' situations that arise unexpectedly during an otherwise routine IVF cycle.

In contrast to the oocyte, cryopreservation of ovarian tissue has gained considerable interest because of its potential application in the preservation of fertility in young women undergoing treatments for cancer (or other disorders that will render them sterile) but may not avoid the re-introduction of malignant cells in autologous transfers. This technology is based on limited animal experiments that have resulted in normal live young. The cryopreservation of ovarian tissue is usually more complicated than cryopreserved gametes or embryos owing to differential cryoprotectant permeation of the tissue. Thus, the choice of cryoprotectant(s) has to be

evaluated for properties that provide effective tissue-specific penetration. To date, no conceptions have been reported in women after transplantation of thawed ovarian tissue fragments or slices, despite indications of transient restoration of menstrual cycles and estradiol production. The problem of ovarian tissue cryopreservation is that only primordial follicles survive after thawing, and methods to obtain fully grown oocytes that are both meiotically and developmentally competent requires a level of extended in vitro growth and development from the primordial stage that has yet to be achieved in the human species. (see Picton, this volume) While this is very promising technology, it is still in the domain of research and therefore, clinical application should be considered in this context.

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CHAPTER NINETEEN

REDUCING THE NUMBER OF EMBRYOS TO TRANSFER AFTER IVF/ICSI

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INTRODUCTION

Recent experience in several countries (Belgium, Finland, Sweden) has shown that the introduction of elective single embryo transfer (eSET) after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) has resulted in a substantial reduction of the twinning rate and an almost complete disappearance of higher-order gestations (multiple pregnancies), without causing the much dreaded decrease in the overall ongoing pregnancy rate of the program. This is mainly the consequence of optimization of the various steps of the IVF/ICSI process: selection of the twin prone patient, ovarian stimulation, oocyte recovery, embryo culture, embryo selection, embryo transfer and a coherent counseling of the patients. The two main determinants have been embryo selection and patient selection, in that order of impact. The degree of reduction in the twinning rate that has been realized to date with relatively simple means and is in the order of ~50%, i.e. a twinning rate of ~30% will come down to ~15%. Since this is just the beginning of the ‘final maturation’ process of IVF/ICSI as a treatment of infertility, the following are some essential questions addressed in this chapter that remain to be solved:

1. What is the most efficient procedure for embryo selection, i.e. the most easily learned, the most swiftly applied, the cheapest, and the most evidence based method to select a single embryo with a high implantation potential?
2. What is the ideal algorithm for integrating the morphological and growth characteristics of embryos into a system of embryo selection?
3. Is it possible to simplify and minimize ovarian stimulation without compromising the essential condition of being able to choose embryos from a cohort available for transfer?
4. Once the first half of the present twinning is avoided, how

- shall we proceed to decrease the ‘remaining half’?
5. How can the increasingly important cryopreservation be optimized in an IVF/ICSI program that has adopted eSET as a standard procedure?
 6. Which strategy should be adopted by each country to involve (financially) both private and public health insurers in a system where reimbursement of an IVF/ICSI treatment is linked with rational limitation in the number of embryos to be transferred?

The first five questions are essentially a matter of laboratory and/or clinical research, the results of which are in principle universally applicable. The latter is a matter of political deliberation between all parties involved. It is a health-economic exercise where clinical decision making models will come into play. Therefore, this exercise will have to be repeated in each individual country. It is only possible if enough validated and reliable data are available regarding the clinical and biological aspects of IVF/ICSI. Nevertheless, early involvement of the health authorities in this complex process of optimizing IVF/ICSI is advisable.

WHAT IS THE HISTORICAL CONTEXT OF THIS APPROACH TO INFERTILITY TREATMENT: THE EUROPEAN EXPERIENCE

In the early days, IVF did not *have* a goal, it *was* a goal in itself. The first years of trial and error, vividly described in Bob Edwards’ and Patrick Steptoe’s “A matter of Life” and the first successes were obtained at a very high price in terms of scientific and clinical endeavors. When reading this first-hand account, it is fascinating to see how the pioneers vacillated between stimulated and spontaneous cycles, between blastocyst transfer (already considered at this early date!) and early cleavage embryo transfer, and whether transfers should involve one or more embryos. Soon enough, IVF became a worldwide phenomenon with an attendant ‘hype’ that stimulated both the research and the commercial aspects of reproductive medicine. IVF gave so much hope to so many childless couples that it was considered good medical practice to maximize the chances for “success” (i.e., *a* pregnancy) often at the price of as much money as required and without serious regard to potential complications for the mother and the newborn(s), especially if they could be handled by intensive care medicine.

When the first retrospective reports of IVF outcomes were published in the early 1990s, (Staessen et al., 1993; Nijs et al., 1993), it was apparent that pregnancy rates did not change if two instead of three embryos were transferred. The focus of attention could and probably should have been

on a reduction in the number of embryos transferred and on better methods to identify *which rather than how many* embryos to replace. Soon after, procedures such as ICSI and MESA/TESE + ICSI made their appearance as powerful tools to solve the many and diverse problems of sperm dysfunction which had been until that time untreatable, except by means of donor insemination, an approach which for many couples was either not an option or their only biological possibility of having a child. Because this treatment was complicated and costly, ICSI actually increased the number of embryos transferred because it was unclear whether the competence of embryos produced by this means was equivalent to those produced by conventional IVF. In many European centers, the transfer of a 'standard' of two embryos has existed for many years and still prevails, although three embryos are often transferred. The evolution of embryo transfer policies in Europe has differed markedly from those in other areas, such as the United States, where it seems as if the goal of many IVF programs has been to tout the number of babies born each year rather than the number of normal singleton or twin pregnancies achieved. As consequence of replacing relatively large numbers of embryos (>6), the last two decades in the US has witnessed what could be termed an epidemic of IVF-related high order gestations that only recently have become so significant an issue as to warrant concerns from governmental and professional organizations. Clearly, some of the issues that have dictated replacement of large numbers of embryos in the U.S. included similar factors that exist elsewhere, such as the high emotional and financial investment of couples and the reality of tough competition between centers. Another important factor that may have contributed to this practice is the lack of confidence on the part of clinicians that embryo competence could be critically assessed in the laboratory at the earliest of development, which may have led to the a 'safety in numbers' approach to clinical IVF. If we take as an example a typical IVF outcome experience where 3 or more embryos are replaced, for each 100 ongoing pregnancies, a twinning rate of 28%, a high-order gestation rate (i.e. triplets and higher) of 4% and a singleton pregnancy rate of 68% are common outcomes. This will results in 136 children born ($56 + 12 + 68$) of whom exactly half belong to a set of multiples! This is the present situation.

It has long been recognized that multiple pregnancy and its obstetrical, neonatal, developmental and financial consequences represents the main iatrogenic complication of in-vitro fertilization and intracytoplasmic sperm injection (IVF/ICSI) (Dhont et al., 1999; Keith and Oleszczuk, 1999; Bergh et al., 2000; Elster, 2000; Schieve et al., 2002), and that urgent action should be taken in order to limit this unwanted state of affairs (EHRE Capri Workshop, 2000). The extremely high incidence of twin pregnancies, varying between 25-40%, constitutes the foremost challenge,

and in some countries, the incidence of triplets remains alarmingly high (Martin and Park, 1999). Several authors have shown that reducing the number of embryos transferred from a standard number of three to two in patients who produce several early cleaving stage embryos to choose from, is not followed by a decrease of the overall ongoing pregnancy rate, but does eliminate most triplets while not diminishing the rate of twin pregnancies (Staessen et al., 1993; Nijs et al., 1993; Fujii et al., 1998; Hu et al., 1998; Templeton and Morris, 1998; Milki et al., 1999; Dean et al., 2000). Although many twins are to be considered a “medical success” and while even triplets and some quadruplets may do “fine”, there is considerable evidence proving beyond doubt that a great number of them, many never heard of or never seen, are stillborn, die in the neo- or perinatal period, or begin their lives with severe retardation, cerebral palsy and other deficits and complications that compromise a normal life, not only for themselves, but for their parents, siblings and for society as a whole. There is no treatment in medicine where a ‘successful’ therapy has a 50% complication rate. It should be realized that a normal, physiological pregnancy in the human female, as opposed to many other species, where multiple gestation is the rule, is a singleton pregnancy.

WHAT IS THE CURRENT AND ESSENTIAL GOAL OF IVF/ICSI?

It has taken twenty-five years before experienced IVF practitioners can clearly state that the ***present and essential goal of IVF/ICSI, as a mature treatment for infertility is to generate an optimal chance for a singleton live birth.*** An optimal chance is quite different from a maximal chance, which is often less than optimal. Maximization mainly looks at the chance for a pregnancy, with patients, physicians and their team “accepting” almost any risk for its occurrence. In contrast, optimization keeps a wise eye on the balance between the end result and the efforts, costs, risks and complications of the treatment. The only way to strive towards optimization is to reduce the number of embryos transferred. A generalized introduction of single embryo transfer is not feasible. The only way, therefore, to realize the essential goal of IVF/ICSI is to identify the putative high competence (PHC) embryo and the twin prone patient.

Since it is clearly established that a standard procedure of limiting the number of embryos to two does not significantly reduce the high rate of twin pregnancy and therefore eliminate the epidemic size of the problem, we have witnessed three approaches to the problem which can be categorized as (1) epidemiological, (2) mathematical and (3) empirical or experimental.

In the first approach, some authors have tried to identify factors that could predict the chance of birth and of multiple birth on the basis of some

key characteristics associated with the background of the patient, to the cycle, and to the embryos available for transfer. In most cases the underlying assumption was to assess those patients in which transfer of *two* embryos would lead to a twin or singleton pregnancy (Templeton and Morris, 1996; Bassil et al., 1997; Commenges-Ducos et al., 1998; Croucher et al., 1998; Minaretzis et al., 1998; Schieve et al., 1999; Terriou et al., 2001; Tur et al., 2001). Few studies have done this type of multivariate analysis with the clear intention of identifying patients suitable for *single* embryo transfer (Strandell et al., 2000).

In the second approach, mathematical models have been developed to try and understand the interrelationship between the chance for pregnancy, multiple pregnancy or no conception as a function of the number and the (theoretical) implantation potential of embryos (Martin and Welch, 1998; Trimarchi 2001).

Both of these retrospective approaches are interesting because in attempting to explain the observed outcomes, they could be useful in actually applying eSET. In this sense, these approaches are preparatory steps required to identify specific clinical and laboratory prerequisites needed to introduce elective single embryo transfer in an ongoing IVF/ICSI program. They are a careful probing of the ‘reality’ of IVF experience.

In the third approach, single embryo transfer is applied in a clinical program and outcome results are analyzed retrospectively. Giorgetti et al. (1995) reported on 957 *compulsory* single embryo transfers, which resulted in a poor outcome as evidenced by an ongoing pregnancy rate of <10% per transfer. The first report of a *noncompulsory* application of single embryo transfer mainly concerned women with a medical contraindication for twin pregnancy (Vilska et al., 1999) with no particular effort made to identify the PHC embryo *prior* to the introduction of SET. Results were very encouraging with an ongoing pregnancy rate of about 30%. Our own approach has been to include a preliminary identification and validation of strict embryo characteristics *prior* to *elective* SET (Van Royen et al., 1999), followed by a prospective, randomized comparison between one versus two selected ‘top quality’ or “putative high competence” (PHC) embryos in twin prone patients (Gerris et al., 1999). Other authors have reported their experience with single versus double embryo transfer, using standard but not strict embryo criteria (De Sutter et al., 2000; Martikainen et al., 2001), and some have suggested a beneficial result when cryopreservation is included in the context of eSET (Tiitinen et al., 2001).

Here, I shall try to show that it is possible, by a judicious application of elective single embryo transfer, to maintain a high stable ongoing pregnancy rate in an IVF/ICSI program, while reducing the total multiple pregnancy rate to half the currently reported incidence of ~30% while at

the same time limiting the occurrence of higher-order gestations to dizygotic triplets. At the outset, two essential points need to be emphasized. First, a constant dialogue between physicians and embryologists is fundamental. Without decreasing the physicians' final responsibility, the embryologists must contribute to the policy a center is adopting. Without decreasing the biologists' 'sovereignty' in the laboratory, the physicians can and must expect from them a constant effort at optimizing laboratory procedures and a constant search for methods that improve embryo culture and selection. At present, many programs perform below their intrinsic potential by not systematically recording embryo characteristics in a prospective way (i.e. creating an embryo data base), by not adopting a validated and strict system of embryo selection, and by performing transfers on a basis of physician availability, rather than on strict timing. I would also include in this list suboptimal embryo transfer techniques including the use of rigid catheters and intrauterine placement of the transfer catheter by feel rather than ultrasonography. Indeed, simply improving these aspects can lead to a significant increase in pregnancy rate. This potential improvement in outcome could be enhanced further by better embryo selection. By adopting the above programmatic suggestions, the essential goal of IVF/ICSI will come closer, even if a wide gap persists between the twinning rate of an optimized IVF/ICSI program and the natural twinning rate of ~1%.

Second, two issues have been confused in a debate that seems to compromise rather than solve the problem of multiple gestation. Some authors have suggested that before introducing single embryo transfer, prolonged culture of embryos to the blastocyst stage is a necessary prerequisite. In doing so, they have confused the problem of how to maximize pregnancy rates with the problem of how to optimize them. Postponement of eSET until this largely academic discussion has been resolved is in my opinion unethical. Reductions in the number of embryos transferred, to a maximum of two, and in many cases to one, should be adopted irrespective of the stage at which the embryo is transferred because in my opinion, the problem of high twinning rates is much more important than the problem of further increasing the chance for a pregnancy. Moreover, it is likely that in an unselected twin prone patient population in which eSET is applied, strict embryo selection at the early stages of embryo development leads to an ongoing implantation rate which is similar for *one* PHC day 3 embryo compared with *one* PHC day 5 or 6 blastocyst. A theoretical rationale and the clinical data to support this argument are given below in an effort to formulate a balanced view regarding this debate.

HOW THE ISSUE OF LIMITED OR SINGLE EMBRYO TRANSFER CAN BE ADDRESSED: CLINICAL AND LABORATORY PREREQUISITES TO ELECTIVE SINGLE EMBRYO TRANSFER

WHAT ARE CURRENT PRACTICES REGARDING EMBRYO TRANSFER?

At present, the most widespread attitude with respect to embryo transfer in a first IVF/ICSI cycle for “young” women is to transfer the two “best looking” embryos, although in many European centers, transfer of three embryos is still routinely performed. The latter attitude has to be considered obsolete. It was shown in retrospective studies that the overall ongoing pregnancy rate of a program was similar in a group of patients in whom the “standard” number of embryos transferred was decreased from three to two (Nijs et al., 1993; Staessen et al., 1993; Tasdemir et al., 1995; Dean et al., 2000) and that it is possible to avoid the large majority of triplet pregnancies by replacing two embryos as the standard of good clinical care. Most convincingly, a large national British study demonstrated that only the multiple birth rate and not the total birth rate increased when three instead of two embryos were transferred in cycles in which more than four oocytes had been fertilised (Templeton and Morris, 1998). In a large analysis of outcome results from the United States, Schieve et al. (1999) found that three factors—patient age, number of embryos transferred, and the ability to select embryos for transfer—had a pronounced effect on the success of an IVF procedure and the risk for multiple birth. Their sample size ($n = 35,554$ embryo transfers) allowed them to explore retrospectively, the interrelationships between outcome and these three factors. Although they acknowledged that they did not have specific laboratory data to classify embryo quality, they observed for women younger than 35 years, that when elective transfers were limited to 2 embryos, with 1 or more embryos cryopreserved, live-birth rates were comparable to those achieved when 3 or more embryos were replaced. However, the twin birth risk was halved and the risk for higher-order pregnancies and births was virtually eliminated. In contrast, women aged 35 to 39 years appeared to receive some benefit from elective transfer of 3 rather than 2 embryos with live birth rates increased by 8 percent. However, this increase was not statistically significant at the 0.05 level. While multiple-birth rates also increased with 3 embryos transferred (29.4%), these risks were much smaller than observed in younger women (20 to 29 years) who received 3 embryos (45.7%). The availability of at least one embryo for freezing was used in this retrospective analysis as an indicator for “elective” transfer (of at least *two* embryos).

From all available evidence, it is now possible to conclude for almost all patients undergoing IVF/ICSI, that reducing the number of transferred embryos from a standard of three to a standard of two does not result in a significant decrease in the overall pregnancy rate, but clearly reduces the potential for triplet pregnancies. While thus giving some assurance that control over triplet pregnancies can be obtained, at the same time these findings also suggest that an unabated high ongoing twin pregnancy rate may be the unavoidable price to pay for an “acceptable” pregnancy rate after IVF/ICSI. In addition, determining the number of embryos to transfer in general is fundamentally different from considering the particular age group of women >38 years of age, twinning not being a major problem in this age group.

The compelling question is whether this reasoning can be extrapolated from two embryos to one embryo, and in this regard, two main variables come into play, the patient-and-her-cycle and *the transferred embryo*. If one were to extrapolate the idea of reducing the number of embryos from a standard of two to a standard of one, it can be anticipated that the overall ongoing pregnancy rate would drop significantly, although there are no data available where such a transfer policy has been strictly adhered to, i.e., where only one unselected embryo is replaced in *all* cycles. Nevertheless, outcome results would certainly be much lower in terms of pregnancies per started cycle than is presently the case with multiple embryo transfers. In addition, when considering the transfer of a single embryo, all systems of “cumulative” embryo scoring are useless because they are based on the transfer of more than one embryo. This is not to say that using such scores does not give interesting information regarding the chance for a pregnancy if more than one embryo is replaced, but they do not permit the choice of *the best embryo* if single embryo transfer is under consideration. To do this, the uses of specific individual embryo characteristics are mandatory.

Therefore, the essence of an eSET approach is the ability to actively select (i.e., elect) the right embryo in the right patient and in the right cycle, hence, the term elective SET or eSET. Apart from this rather evident reasoning, not every program may be ready for the introduction of eSET. Some prerequisites have to be fulfilled; both on the laboratory and on the clinical level, as well as during the important phase where both are inextricably combined at embryo transfer. Given the fact that an ongoing pregnancy rate of $\sim 30\%$ in first cycles in women <38 years of age is currently expected, no program director will adopt eSET if the program struggles with a relatively low pregnancy rate. Moreover, the twin pregnancy rate must be compellingly high before all participants in a programme truly support eSET. It is difficult to express this in a figure, but multiple pregnancy rates of 25% for twins and 3-4% for triplets are common in programs with a “standard” two embryo transfer policy.

Although this figure is staggering and although it is almost inconceivable in any other branch of medicine to have an iatrogenic complication rate in more than 50% of treatment cases, many programs do not consider this argument sufficiently compelling to adjust their transfer policy.

WHY SHOULD ESET BE CONSIDERED?

It is not my intent to convince anyone of anything. It is clear as glass that there are two different questions that have to be answered independently and in the right order. The first is do I *want* to reduce my twinning rate (and as a sequel, the higher order multiple pregnancy rates) or do I not want this? The second is *if* I want it, how do I proceed?

Many questions and considerations can be formulated regarding the first question: are twin pregnancies a complication (yes, they are, because all published neonatal and obstetrical data attest to this); are twins wanted and desired (no, there are not many patients actually expressing a desire for twins); who is to decide (the doctor or the patient?); and there are other related questions. If a particular physician and/or his/her team decides there is no place for eSET because neither the patients request it or the doctor wants it (each of them for their own – morally justifiable? – reasons), then the second question is irrelevant and the data provided in the effort to answer it will not be able to convince a mind that is already convinced of the opposite, namely that we have to live with a high twinning rate. However, if it is decided that twins should be prevented to some – a matter of clinical judgement – extent, the second question becomes relevant. Only time will tell what is good and what is the better clinical practice. In the present contribution, I assume that there is some desire to strive towards infertility treatment as a mature form of alleviation of the sufferings of human infertility and not as a purely business-like enterprise with a goal that goes beyond that of applying the action-science in which medicine is evidence based. That means, we can assume that one has reached the stage of deplored the high twinning rates up to a point where action to avoid it is morally right.

In putting things in this way, I am trying to avoid falling in the trap of moralising. No one should be compelled to accept or apply eSET. But people should be informed, counselled and supported, not in the least financially, to have low threshold access to treatments that should essentially aim at the birth of a healthy singleton.

CAN PATIENTS SUITABLE FOR ESET BE SELECTED?

Several authors have contributed to our understanding of the clinical circumstances in which one should be inclined to *limit* the number of embryos replaced. It should be stressed that for most of these authors, “to

"limit" still means replacing two embryos instead of three. In contrast, for these patients, we advocate the replacement of only one embryo. Several background characteristics of the patients as well as cycle-specific characteristics have been found to correlate with the chance of a birth and/or to the chances for multiples (Templeton et al., 1996; Bassil et al., 1997; Minaretzis et al., 1998; Templeton and Morris, 1998; Strandell et al., 2000; Tur et al., 2001). While the conclusions of most of these reports are similar, of all factors examined, the most important one is female age. With respect to multiple birth, a significant contribution was made by Templeton and Morris (1998) who found the following factors correlate with outcome: age, tubal infertility, four or more previous IVF/ICSI attempts, duration of infertility, previous live birth (either natural or with IVF), but significantly, not the number of fertilised eggs obtained or number of embryos transferred. The authors constructed a table to aid in clinical decision making, in which these factors were allotted a certain weight. This approach seems to identify patients with relatively high or low probabilities of achieving a live birth, as well as those at risk for a multiple birth. Their main point was that no increase in birth rate is obtained by systematically transferring three embryos when compared with two. At this time (1997-1998), this conclusion provided an important insight because it was associated with the prevention of most triplet pregnancies. However, it was premature to suggest that transfer of a single embryo was acceptable.

With respect to the identification of patients at risk for twin pregnancy, a significant contribution was made in a Swedish study (Strandell et al., 2000). In a retrospective study of 2107 IVF cycles in which two embryos were transferred, these authors examined which factors may correlate with multiple births. From this data, they tried to identify a subset of patients at significantly higher risk of multiple birth in whom one-embryo transfer could be applied without impairing the birth rate. They used multivariate analysis to assess similar potential risk factors that included the following parameters: (a) patient history: age, prior natural and IVF pregnancies and births, (b) *IVF cycle characteristics* such as FSH dosage and duration, (c) number of retrieved oocytes, fertilization method - IVF or ICSI, (d) number of fertilized oocytes, fertilisation index (proportion of fertilised oocytes), (e) number of "good quality" embryos available for transfer, day of transfer (day 2 or 3 after oocyte retrieval), (f) number of "good quality" embryos transferred and (g) the number suitable for freezing. What characteristics defined "good quality" was not specified, nor were inclusion criteria for freezing stated. All women were younger than 40 years of age. The following factors correlated independently with singleton and multiple births, adjusted for the number of previous IVF cycles given the fact that the chance for pregnancy decreases with the rank of the trial above two trials: age, initial daily dose of FSH, total dose of

FSH, tubal versus other causes of infertility, number of good quality embryos available, number of good embryos transferred, number of embryos available for freezing.

From these studies several conclusions can be drawn. First, positive outcomes when tubal infertility is involved (which was the original indication for IVF) are significantly less frequent than with other indications. Second, the risk for multiple pregnancy and the chance to become pregnant are independently related to age. Older women have a reduced chance for pregnancy but the proportion of such patients with multiple gestations is *not lower* than observed in young women (in contrast to the situation that occurs after natural conception). Third, the odds ratios of the factors examined are as a rule close to 1, suggesting a low impact of each individual variable. It is important to keep this in mind when looking at the importance of embryo characteristics. Fourth, whereas the U.K. report (Templeton and Morris, 1998) focused on the two-or-three embryos issue, the Swedish (Strandell et al., 2000) study tried to evaluate what would have happened if in certain cycles, only one embryo was transferred. The latter authors offered a theoretical calculation of birth and multiple birth rates using the assumption that female age and the quality of the embryos were the two single most important factors that can predict these rates. They concluded that the rate of multiple birth could be reduced from 26% to 13% of all births if eSET is performed in selected cases. They predicted that the total birth rate would drop from 29% to 25% but would be compensated by a slight gain with pregnancies occurring after replacement of frozen/thawed embryos. The authors suggested that in a true one-embryo program, implantation rates would probably still be higher, because more effort would be placed on optimal selection of high-implantation potential embryos. As discussed below, we confirmed this notion in a prospectively designed impact study. Other authors have singled out additional factors that have an impact on the probability of achieving a live birth after IVF/ICSI. Table 19.1 provides an overview of factors that have been found to correlate significantly with the achievement of a live singleton or multiple birth after IVF/ICSI. In addition, it was demonstrated that patients with a non-ongoing conception in their first IVF/ICSI cycle had a better prognosis for a live birth in their second attempt than patients without evidence of implantation (Croucher et al., 1998).

TABLE 19.1. CORRELATIONS BETWEEN CLINICAL FACTORS AND THE OUTCOME OF IVF/ICSI.

CLINICAL FACTOR	CORRELATION
Female age	Negative – decline >35 years
Tubal infertility	Negative – strong
Unexplained infertility	Positive – weak
Previous attempts of IVF if >2	Negative - strong
Duration of infertility	Negative – weak
FSH initial daily dose	Negative – strong
FSH total dose	Negative – weak
Dose of gonadotropin per oocyte collected	Negative - strong
Use of donor eggs <30 years	Positive – strong
Use of donor eggs at 30 years and >	Negative – weak
No. of good embryos available	Positive – weak
No. of good embryos transferred	Positive – strong
No. of embryo fit for freezing	Positive – weak
Non-ongoing conception in a first IVF-cycle	Positive – strong
Day 3 FSH and E ₂ levels	Negative - strong
NOT CORRELATED	
Female weight	
Primary or secondary infertility	
Method of fertilization (IVF or ICSI)	
Previous pregnancy	Controversial
Previous livebirth	Controversial
Duration of ovarian stimulation	
No. of eggs retrieved	Controversial
No. of fertilized oocytes	
Proportion of fertilized oocytes	
Day of transfer (day 2 or 3)	Controversial
Stage of embryo transfer (2PN, 2-3, blast)	Controversial
Previous attempts of IVF if 1-2	Controversial
Assisted hatching	Controversial
CORRELATED WITH MULTIPLE BIRTH	
Female age	Positive – strong
Previous IVF-cycles	Negative - strong
No. of eggs and embryos transferred	Positive - strong

Weak correlation = odds ratio near 1.00; strong correlation = odds ratio <0.7;
controversial = contradicting reports.

CAN OVARIAN STIMULATION BE OPTIMISED FOR IVF/ICSI?

Recently, two cost-effectiveness studies based on a decision-analysis model (Sykes et al., 2001; Daya et al., 2001) concluded that in addition to the increased effectiveness of rFSH in Assisted Reproductive Technology (ART), its use is also more cost-effective and more efficient than natural gonadotropin(s) derived from urine. However, this conclusion is controversial and others have reported that in routine ovulation induction cycles, 'generic' human menopausal gonadotropins (hMG) do not adversely affect pregnancy rates in comparison to more costly recombinant or highly purified FSH and is therefore an appropriate (Gleicher and Karande, 2000). Although excessive LH secretions present in some disorders can be detrimental to reproductive function, this is not applicable in ovulation induction with hMG because this menotropin does not increase daily plasma LH levels. The results of ovulation induction with hMG or FSH-only regimens did not differ in studies conducted in patients with polycystic ovary syndrome and in most studies conducted in ovulatory women undergoing assisted reproductive techniques; conversely, hMG was clearly superior to purified FSH for the treatment of hypogonadotropic hypogonadism. Thus, low but detectable LH concentrations positively influence the outcome of ovulation induction in patients with ovulatory disorders in general and in most women undergoing ART in particular (Filicori, 1999). When administered at similar dosages, hMG appears to be moderately, but significantly, more effective than highly purified FSH at inducing ovulation in GnRH agonist-suppressed women scheduled for intrauterine insemination (Filicori et al., 2001). In IVF, a prospectively randomised comparison showed the same result (Ng et al., 2001). An increased risk of early pregnancy loss by profound suppression of LH in women undergoing IVF after FSH-alone stimulation has been documented (Westergaard et al., 2000). Another study (Strehler et al., 2001) and another meta-analysis (Agrawal et al., 2000) both showed that in the "long and short GnRH agonists protocol" of IVF, rFSH and hMG were equally effective in achieving ovarian stimulation, and there were no significant differences in clinical pregnancy rates per cycle. Taking this reasoning one step further, some authors have suggested the possibility that a substantial number of normogonadotropic women are profoundly desensitised by standard GnRH agonist suppression, and could benefit from the addition of LH to their stimulation protocol (so-called 'sweetened cycles'). Because there is no reliable or cost-effective method to detect women who will need additional LH administration, it seems practical to systematically add LH to the ovarian stimulation protocols (Lévy et al., 2000). Whether this means reverting to the urinary gonadotropins or following the technology-

driven development of combining recombinant FSH and LH (rLH) products remains to be determined. Some studies have already suggested that the addition of rLH to an rFSH-stimulation protocol improves IVF outcome (Laml et al., 1999; Phelps et al., 1999; Gordon et al., 2001) including blastocyst implantation rates (Schoolcraft et al., 1999). What does seem beyond doubt is that some patients need more basal LH than available if they are severely suppressed using GnRH-agonists, and that the presence of low amounts of LH seems mandatory, perhaps even sufficient to stimulate follicular growth, once initiated beyond a certain point.

CONSIDERATIONS ON OVARIAN STIMULATION FOR IVF/ICSI WITH RESPECT TO THE PREVENTION OF MULTIPLE PREGNANCY?

One of the major questions for clinical research in the years to come is whether it is still necessary to maximise ovarian stimulation to produce large numbers of antral follicles, especially if elective single embryo transfer becomes the gold standard in IVF cycles where a high competence embryo, which I term the top quality embryo can be predictably identified? At present, at least "several" mature oocytes are needed to obtain top-quality day 3 embryos or good quality day 5/6 blastocysts, since such embryos occur in approximately 2/3 and 1/3 of all cycles, respectively, with current high-dose regimens of ovarian stimulation. If the number of oocytes retrieved is decreased due to minimal (so-called patient- "friendly") stimulation, so presumably will the number of excellent embryos available for transfer regardless of the stage selected for transfer, unless stimulation regimens are devised that yield fewer but mostly mature metaphase II oocytes. It cannot be excluded that current ovarian stimulation by itself has a detrimental effect on some oocytes, or that current schemes are actually "overshooting" by producing no more good quality oocytes and embryos than might be the case with minimized stimulation. At the far end of this line of thought, single-oocyte recovery in natural or natural supported cycles might well produce acceptable numbers of good quality embryos to perform "compulsory" single embryo transfer at a much reduced effort and cost for the patient. Although clinical research focussed towards this possibility is ongoing, current data is insufficient to support this approach (Pelinck et al., in press).

A promising breakthrough in the mode of ovarian stimulation comes from the recent studies of Filicori et al (2002a,b) who used the following approach: following priming with human rFSH they progressively lowered the dose, replacing it with increasing levels of low-human chorionic gonadotropin (hCG). This procedure did not negatively affect

larger follicular development but did reduce the occurrence of small preovulatory follicles; daily administration of 200 IU hCG was capable of maintaining folliculogenesis and steroidogenesis even when rFSH was completely discontinued. Although this approach needs confirmation, it seems to combine the best of two worlds: a fair number of mainly mature oocytes from large follicles and a low risk for Ovarian Hyperstimulation Syndrome (OHSS), the occurrence of which is largely related to the presence of small and intermediate sized follicles. At present, it seems prudent not to combine the decision to introduce eSET in an ongoing IVF/ICSI program with the decision to proceed towards minimal ovarian stimulation.

IS EMBRYO TRANSFER THE FORGOTTEN PHASE OF IVF/ICSI?

With respect to outcome, an optimised embryo transfer procedure is perhaps the most influential of all phases of the IVF/ICSI process. It is the point where clinical decisions and laboratory efforts often provide the patient with the first realistic expectation of outcome. It is the phase where small details may have significant effects, as elegantly described in a recent prospective trial by van Weering et al. (2002). Practice protocols and procedures can vary widely between centers, and the relative importance of specific factors that influence outcome at transfer have been identified in a large survey analysis in the UK (Salha et al., 2001). In a prospective randomised trial, some authors reported an increased pregnancy rate with ultrasound-guided embryo transfer (UGET) as compared to the so-called clinical-touch transfer (Coroleu et al., 2000; Wood et al., 2000; Prapas et al. 2001). The underlying rationale for UGET probably relates to embryo(s) deposition at a location within the uterine cavity that may be optimal with respect to the fundus and with this type of visualisation, transfers may be less traumatic than by feel alone. Waiting for thirty seconds before withdrawing of the catheter showed no significant improvement over immediate withdrawal (Martinez et al., 2001). Prolonged bed rest after transfer is not necessary (Sharif et al., 1995a) nor does systematic mock embryo transfer (Sharif et al., 1995b). It has been demonstrated that the use a soft catheter improves results and that the impact of using a soft catheter on the ongoing pregnancy rate increases when the *a priori* chance of pregnancy increases (van Weering et al., 2002). The impact of transfer media type, volume and rate of delivery remains to be determined with well-designed multicenter trials. Conversely, poor transfers or poor transfer techniques will have a negative effect, regardless of all the positive selection criteria that have been applied up to this phase of treatment. The effect of transfer technique appears to be strongest in first treatment cycle (van Weering et al., 2002).

Perhaps the most important of all is the ‘provider-effect’. Continuous monitoring of the outcome of IVF/ICSI cycles as a function of which embryologist and clinician was involved in the production and transfer of embryos is an essential part of quality assessment of *any* programme. However, it has been shown that even after correction of attitudes and practices, differences that can have important consequences for outcome tend to persist (Karande et al., 1999; Hearn-Stokes et al., 2000), and as such, these tendencies need to be identified and addressed. In our center, we found an inverse correlation between the depth of introduction of the outer catheter and the chance to obtain a pregnancy. Others also found the optimal distance to insert an embryo to be approximately 1.5 cm from the fundus (Coroleu et al., 2002).

THE CURRENT STATUS OF ESET

PUBLISHED DATA ON THE ELECTIVE TRANSFER OF A SINGLE DAY 3 EMBRYO

The first paper to mention results with *compulsory* single embryo transfer is from a French group, who performed a retrospective analysis of 957 transfers in cycles where only one embryo was available (Giorgetti et al., 1995). A correlation was found between certain embryo characteristics and the implantation rate. Although the overall results of these cSET-cycles were expectedly poor, they illustrate the correlation between embryo morphology and implantation potential. As an additional finding, results plummeted in women > 38 years of age. Others have published similar results after compulsory SET (Elsner et al., 1997). A Finnish group was the first to report a retrospective cohort study in which 910 embryo transfers were studied in which 168 involved a single embryo: only one embryo was available in 94 transfers and for 74 transfers, SET was elective, i.e. there were at least two embryos available but only one was transferred because of patient’s wish, risk for OHSS, or purely medical reasons (cervical incompetence, uterine malformation, diabetes mellitus or an indication for prenatal diagnosis) (Vilska et al., 1999). The results are shown in Table 19.2. The details of embryo assessment were as follows: fertilization was determined by the presence of pronuclei at 16-to-19 hours after insemination/injection. All embryos containing two clearly visible pronuclei were combined in one fresh 10 μ l microdrop of Ménézo B2 medium (max. 10 oocytes/drop) and cultured for an additional 24 hours. The next day, 40-43 hours after insemination/injection, the embryos were separated and each transferred to a 10 μ l drop of medical M3 Medium for a further 24 hours of culture. Every embryo was examined for the total number of cells, the presence of anuclear fragments and multinucleated blastomeres. From the moment day 2 embryo criteria

were recorded, embryos were cultured separately. On day 3, 64-67 hours after IVF or ICSI insemination, embryos were reevaluated. All transfers were performed on day 3, 70-to-72 hours after oocyte retrieval. Supernumerary embryos were frozen.

The characteristics of these 46 implanting embryo leading to 23 healthy twin children were absence of multinucleated blastomeres, 4 or 5 blastomeres on day 2, a minimum of 7 cells on day 3 and a maximum of 20% anucleate fragments. In order to validate these criteria, allowing an embryo to be considered a putative high competence embryo, a subsequent series of 400 IVF/ICSI cycles was retrospectively analysed, in which only women <38 years of age had multiple pregnancies: for 221 transfers of 2 embryos, multiple pregnancies occurred in 45 of 116 (39%) patients, and after 77 transfers of more than 2 embryos 11 out of 31 (35%) of patients. We applied the above mentioned criteria to the 221 two embryo transfers: 106 transfers with 2 top embryos resulted in 65 (63%) ongoing pregnancies with 37 (57%) twins, 65 transfers with 1 top embryo and one non-top embryo resulted in 38 (58%) ongoing pregnancies with 8 (21%) twins. In the two-embryo transfer group without any top quality embryos transferred, there were 12 (23%) ongoing pregnancies in 52 transfers, but no twins. The ongoing implantation rates were 49%, 35% and 12% (Table 19.3). Implantation rates were highest when the embryos were at the 4-to-5-cell stage on day 2 and at the 6-to-8-cell stage on day 3. The implantation rate was ~30% in the group where elective SET was performed. This figure has to be kept in mind and compared to an ongoing pregnancy rate of ~40% observed when the presence or absence of multinucleated blastomeres is added to the selection criteria (see below).

TABLE 19.2. PREGNANCY RATE AND MULTIPLE PREGNANCIES FOLLOWING THE TRANSFER OF ONE OR TWO EMBRYOS AFTER IVF/ICSI

Type of treatment	Transfers (n)	Clinical PR/ET (%)	Twin PR/clinical Pregnancy (%)
Transfer of 2 Embryos	742	218 (29.4)	52 (23.9)*
Transfer of 1 embryo (compulsory)	94	19 (20.2)	0
Transfer of one embryo (elective)	74	22(29.7)	0

*Including one set of dizygotic triplets
(adapted from Vilska et al., 1997)

Whereas several authors have examined clinical profiles as the primary way to approach the problem of multiple pregnancy, others have looked at the characteristics of the embryos considered for transfer. In the first approach, the clinical profile of the "twin prone" patient is considered to be a young (initially: <34 years of age) woman in her first IVF/ICSI cycle. Approximately 80% of all twins occur in this age group during the first two cycles of treatment. Clearly, this is the group where eSET will give the best result. What is needed for the second approach are strict and validated criteria to identify embryos with optimal implantation potential. Estimations of embryo implantation potential have always been based on performance characteristics that include morphology and rates of cell division that have been largely difficult to validate. We have taken the view that the introduction of eSET in an ongoing IVF/ICSI program should be preceded by an intensive effort to define characteristics of high implantation embryos defined as strictly as possible. Moreover, these characteristics should be non-invasive, intrinsically easy to observe and learn, and not add additional expense to the IVF procedure. We derived such characteristics from a retrospective analysis of a number characteristics of day 3-embryos with a 1:1 known outcome, *in casu* 46 embryos resulting in 23 ongoing twins (Van Royen et al., 1999).

The details of embryo assessment were as follows: fertilization was determined by the presence of pronuclei at 16-to-19 hours after insemination/injection. All embryos containing two clearly visible pronuclei were combined in one fresh 10 μ l microdrop of Ménézo B2 medium (max. 10 oocytes/drop) and cultured for an additional 24 hours. The next day, 40-43 hours after insemination/injection, the embryos were separated and each transferred to a 10 μ l drop of medical M3 Medium for a further 24 hours of culture. Every embryo was examined for the total number of cells, the presence of anuclear fragments and multinucleated blastomeres. From the moment day 2 embryo criteria were recorded, embryos were cultured separately. On day 3, 64-67 hours after IVF or ICSI insemination, embryos were reevaluated. All transfers were performed on day 3, 70-to-72 hours after oocyte retrieval. Supernumerary embryos were frozen.

The characteristics of these 46-implanting embryos leading to 23 healthy twin children were *absence of multinucleated blastomeres, 4 or 5 blastomeres on day 2, a minimum of 7 cells on day 3 and a maximum of 20% anucleate fragments*. In order to validate these criteria, allowing an embryo to be considered a putative high competence embryo, a subsequent series of 400 IVF/ICSI cycles was retrospectively analysed, in which only women <38 years of age had multiple pregnancies: for 221 transfers of 2 embryos, multiple pregnancies occurred in 45 of 116 (39%) patients, and after 77 transfers of more than 2 embryos 11 out of 31 (35%) of patients. We applied the above mentioned criteria to the 221 two

embryo transfers: 106 transfers with 2 top embryos resulted in 65 (63%) ongoing pregnancies with 37 (57%) twins, 65 transfers with 1 top embryo and one non-top embryo resulted in 38 (58%) ongoing pregnancies with 8 (21%) twins. In the two-embryo transfer group without any top quality embryos transferred, there were 12 (23%) ongoing pregnancies in 52 transfers, but no twins. The ongoing implantation rates were 49%, 35% and 12% (Table 19.3).

TABLE 19.3. SUBGROUP OF 221 TRANSFERS OF 2 EMBRYOS SPLIT INTO THREE GROUPS ACCORDING TO WHETHER 2, 1 OR NO TOP QUALITY EMBRYOS WERE TRANSFERRED

	2/2 top embryos	1/2 top embryo	0/2 top embryos
N embryos transferred	2	2	2
N transfers	104	65	52
N ongoing pregnancies (%)	65 (63)	38 (58)	12 (23)
N singletons (%)	28 (43)	30 (79)	12 (100)
N twins (%)	37 (57)	8 (21)	0
N triplets (%)	0	0	0
Ongoing implantations (%)	102/208 (49)	46/130 (35)	12/104 (12)

(adapted from Van Royen et al., 1999)

This analysis showed that the implantation rate of embryos with morphological characteristics derived from the original analysis of 23 twins resulting from 23 double embryo transfers is the highest and we considered such embryos to be PHC embryos. Therefore, this second retrospective analysis suggested that these embryo criteria should allow a consideration of single embryo transfer with an acceptable pregnancy rate when a single top quality embryo is available. It should be remembered that "traditional" embryo selection criteria look only at the cleavage speed (considering the quickest dividing embryos as the one to consider for transfer) and at fragmentation (considering embryos with the least fragmentation as the ones to consider for transfer) and do not take into consideration the systematic search for multinucleation in all the blastomeres.

Only one embryo back if they wanted this; these patients were evidently not part of the randomization, but the outcome of these cycles was also recorded (Table 19.4; group D). Supernumerary embryos with > 7 blastomeres on day 3 were frozen. The study was limited to the first treatment cycle. The aim of the study was to obtain data on the ongoing

implantation, pregnancy and multiple pregnancy rates in women receiving either one or two top quality embryos. Results are shown in Table 19.4.

We concluded from these data that using strict embryo criteria prior to eSET, an ongoing pregnancy rate (OPR) similar to the one observed in normal fertile couples can be achieved after IVF/ICSI, while limiting the twin pregnancy rate to its natural incidence of <1% of all ongoing pregnancies. Coetsier and Dhont (1998) were another Flemish group to test the notion of eSET on the basis of theoretical speculations similar to those described by Strandell et al (2000). Their clinical experience in a matched case-control study was reported during an ESHRE Workshop held in Antwerp in May of 2000 and is summarized in Table 19.5. Their results are comparable to ours with respect to OPR of 36% for a single 'excellent' embryo.

As a consequence of eSET, there should be more embryos available for freezing. The potential negative effect eSET might have on the overall pregnancy rates could be compensated for by pregnancies obtained with thawed embryos, a possibility that has been confirmed by Tiitinen et al. (2001). These authors performed a retrospective cohort study of all their elective single embryo transfers during 1998-1999. All transfers were performed on day 2 after oocyte retrieval and in all cases at least one embryo was frozen. A total of 127 eSETs were performed with a clinical pregnancy rate of 38.6%. The highest implantation rate was obtained with 4-cell embryos with <10% fragmentation, but multinucleation was not included in their assessment scheme. The result of this study, shown in Table 19.6 involved 129 frozen-thawed embryo transfer cycles that included 46 with one and 83 with two embryos transferred. Outcome findings demonstrate the same clinical implantation rate, 38.6%, for embryos judged to have the highest implantation potential in fresh day 2 transfers. These authors also obtained data on the cumulative pregnancy rate after transfer of fresh and frozen-thawed embryos in-patients that underwent eSET (Table 19.7).

These data demonstrate that a well performing cryopreservation program is an important prerequisite prior to introducing eSET. What is "lost" in terms of ongoing pregnancies due the application of eSET in highly twin prone patients seems to be gained back by cryopreservation. Twins "lost" in the fresh cycle of single embryo SET return as born after freeze/thawing. Another Finnish group performed a multicentric prospective comparative study in 144 women who were randomised to receive either one or two embryos (Martikainen et al., 2001). No mention is made in this study of the precise embryo selection method. First cycles were excluded and all patients had to have at least four good quality embryos in order to be enrolled. A total of 1301 cycles fulfilled the inclusion criteria of which 144 patients agreed to participate in the same

period, 1476 cycles involved patients who did not fulfil the inclusion criteria.

TABLE 19.4. COMPARATIVE OUTCOME OF SEVEN GROUPS OF PATIENTS

	A (SET)	B (DET)	C (NR)	D1 (eSET+)	D2 (eSET-)	E (NA)	F (NE)
N cycles (transfers)	29	36	76 (69)	36	15 (13)	82 (78)	505 (474)
Mean n embryos replaced	1	2	1.72	1	1.53	1.89	2.35
Positive hCG	18	28	27	16	2	42	211
Clinical pregnancies	14	26	26	13	2	36	167
Ongoing pregnancies	11 ^a	24	24	13	2	32	140
Multiple pregnancies	1 ^a	6	12	0	0	13 ^b	48 ^b
Conception rate (%)	62.1	77.8	39.1	44.4	13.3	53.8	44.5
Clinical pregn. rate (%)	48.3	72.2	37.7	36.1	13.3	46.1	35.2
Ongoing pregn. rate (%)	37.9	66.7	34.8	36.1	13.3	41	29.5
Multiple pregn. rate (%)	9.1	25	50	0	0	40.6	34.3
Ongoing implant. rate (%)	37.9	47.1	27.5	36.1	8.7	29.7	17.4

^a One monozygotic twin

^b Two dizygotic triplets

Group A (n=26; single embryo transfer, SET), group B (n=27; double embryo transfer, DET), group C (n=57; recruited, did not produce two top quality embryos but received all two best embryos except three patients, NR), group D1 (n=11; elective single embryo transfer, received one top embryo, eSET+), group D2 (n=6, elective single embryo transfer, did not receive one top embryo, eSET-), group E (n=67; did not agree to participate, NA) and group F (n=352, all other cycles, not recruitable by inclusion criteria, NE) (adapted from Gerris et al., 1999).

The one-embryo group had 32% pregnancies; the two-embryo group 47%. In the one-embryo group, an additional 7 ongoing pregnancies resulted after transfer of frozen-thawed embryos, resulting in a total of 31/74=42% OPR per started original cycle. In the two-embryo group, only one additional ongoing pregnancy resulted from freeze/thawing, increasing the OPR per original cycle to 48.6%. Both figures are not significantly different.

TABLE 19.5. PREGNANCY RATES WITH RESPECT TO EMBRYO QUALITY IN GHENT, BELGIUM

	ESET excellent	ESET good	EDET 2 excellent	EDET 1 excellent + 1 good	eDET 2 good
N transfers	77	44	546	287	364
PR ^a (%)	42	45	40	47	43
Ongoing PR ^b (%)	36	40	31	35	37

All figures not significantly different.

(adapted from ESHRE Report, 2000)

^a All conception cycles with a serum HCG > 1000 IU/ml

^bOngoing clinical pregnancy rate per started cycle

TABLE 19.6. PREGNANCY RATE AND MULTIPLE PREGNANCIES FOLLOWING THE TRANSFER OF ONE OR TWO EMBRYOS AFTER IVF/ICSI

Type of transfer	N transfers	Clinical PR per transfer (%)	Delivery rate per transfer (%)	Twin pregnancy Rate per Delivery (%)
2 embryos	517	203 (40)	160 (30.9)	42/160 (26.2)
Only 1 Embryo	94	17 (18.1)	13 (13.8)	1 /13 (7.7)
Elective 1 Embryo	127	49 (38.6)	34 (26.8)	1/34 (2.9)

(adapted from Tiitinen et al., 2001)

In conclusion, it can be said that several authors, especially from Belgium and Finland, have shown that both in highly selected and in randomly chosen patients, elective single embryo transfers yields excellent results when applied in women < 34 years of age in their first or second IVF/ICSI cycle, and that cryopreservation plays an important role in overall outcome results.

TABLE 19.7. CUMULATIVE PREGNANCY RATE AFTER TRANSFER OF FRESH AND FROZEN-THAWED EMBRYOS IN SUBJECTS TREATED WITH ESET

Type of transfer	N transfers	Pregnancy rate (%)	Delivery rate (%)	Twins (%)
Fresh embryo transfer	127	49 (38.6)	34 (26.8)	1 (2.9)
Frozen embryo transfer	129	39 (30.2)	32 (24.8)	4 (12.5)
One embryo	46	8 (17.4)	5 (10.9)	0
Two embryos	83	31 (37.3)	27 (32.5)	4 (14.8)
		78 (62.4)	66 (52.8)	5 (7.6)

(adapted from Tuutinen et al., 2001)

PUBLISHED DATA ON THE ELECTIVE TRANSFER OF A SINGLE EMBRYO AT OTHER STAGES OF DEVELOPMENT

OOCYTE SELECTION

Although no *clinical* studies have been performed using characteristics of the oocyte or maturing follicles, such as follicular vascularization, as the criterion for subsequent embryo selection and the prospective use of follicular vascularity for embryo selection in a clinical IVF program has been reported by Bhal et al. (1999a; see Gregory this volume). The following quality-related factors have been proposed: (1) morphology of the cumulus-coronal complex (Ng et al., 1999), (2) oocyte morphology, including apparent density of cytoplasm, (3) the presence of cytoplasmic vacuoles or extracellular fragments (Loutradis et al., 1999), and (4) morphology of the first polar body (Ebner et al., 1999). Each of these appears to have an impact on pregnancy outcome. ATP content of oocytes and the dissolved oxygen content of preovulatory follicular fluid have been implied as epigenetic influences on oocyte development (Van Blerkom et al., 1995, 1997, and 1998).

Table 19.8 offers a survey of the different methods currently used in clinical IVF to select embryos for transfer at different stages of preimplantation development. Methods are ranked according to the developmental stage at which selection took place and for each, the maximal implantation rate (for optimal embryos) reported by the authors is given. It should be emphasised that this overview takes into account the *actual* criteria used for embryo selection and not the *correlated* findings. For example, in the Scott et al. (2000) paper, the authors *actually used* “traditional” day-3 criteria to choose the embryos to transfer (i.e. not looking at multinucleation), but they *correlated* implantation rates

retrospectively with the 2PN-status of the zygotes. There is difference between showing a correlation between two things and *prospectively using* that correlation to build a routine (one-embryo) transfer strategy.

SINGLE ZYGOTE (2PN-EMBRYO) TRANSFER

Some authors have reported on the successful use of morphological characteristics of 1-cell embryos to identify those with high competence. This approach has been used in clinical practice (Scott and Smith, 1998; Ludwig et al., 2000a and 2000b), and is largely weighted towards specific patterns of pronuclear development and morphology (Tesarik and Greco, 1999). Tesarik et al. (2000) reported that transfer of a single cleavage stage embryo that showed a Type 0 pronuclear pattern at the 1-cell stage resulted in an implantation rate of 30% per transfer, with high twin rates when two pattern 0 embryos were transferred. This finding suggests that selection at the pronuclear stage can lead to the application of a single embryo policy and optimise the selection of embryos for transfer and cryopreservation. In the light of an ongoing implantation rate of ~35% using day 2 and 3 embryo criteria, it remains to be established whether pattern 0 zygotes coincide with top quality day 2 and 3 embryos, or whether there is an additional advantage in combining day 1 with day 2/3 criteria.

SINGLE BLASTOCYST TRANSFER

Many clinicians have led to believe that elective single embryo transfer necessarily implies prolonged culture of the embryos to the blastocyst stage, the rationale being that the longer an embryo persists in vitro, the more likely it is that it will have a high implantation potential. However, at the time of writing no peer-reviewed publications are available containing *data* on single blastocyst transfer as a standard policy in an otherwise unselected twin prone patient population. Single blastocyst transfer is suggested by several authors on the basis of the retrospective analysis of excellent results obtained after transfer of two blastocysts in selected groups of good prognosis patients, e.g. patients in whom at least ten follicles of >12 mm diameter were present by sonography at the time of HCG administration or other forms of positive patient selection, but not in the general population (Gardner et al., 1998; 2000; Milki et al., 1999; Schoolcraft et al., 1999).

There are three prospectively randomised comparisons of blastocyst transfer (Coskun et al., 2000; Rienzi et al., 2002; Utsunomiya et al., 2002). The first paper compares 101 transfers of two day-3 embryos with 100 transfers of two blastocysts. The pregnancy rate was exactly the same in both groups (39%); the implantation rate was 21% for day 3 and 24%

for day 5, differences that were not statistically significant. Moreover, the study showed that selection of patients had an important affect on the results: for patients with a high number of normally fertilized embryos or with a fair number of 8-cell embryos on day 3, the pregnancy and implantation rates increased. Furthermore, patients who had poor embryos on day 3 still had a high pregnancy and implantation rate if the available

TABLE 19.8. COMPARATIVE OVERVIEW OF DIFFERENT METHODS OF EMBRYO SELECTION

		Max. preg. Rate	Embr/ Transfer	Max. Imp.rate
Ebner et al., 1999	Day 0			0%
Ludwig et al., 2000	Day 1	22%	3	
Giorgetti et al., 1995	Day 2	15,6%	1	16%
Ziebe et al., 1997	Day 2	49%	2	26%
Scott and Smith 1998	Day 2 + zygote	65%	3,7	28%
Tesarik et al., 2000	Day 2 + zygote	44,8%	1,93	30%
Martikainen et al., 2001	Day 2	32,4%	1	32%
Hardarson et al., 2001	Day 2	52,9%	1,98	36%
Vilska et al., 1999	Day 2	35,8%	1	36%
Desai et al., 2000	Day 3	41,9%	3,4	18%
Huisman et al., 2000	Day 3	26,4%	1,9	18%
Coskun et al., 2000	Day 3	39%	2	21%
Wittemer et al., 2000	Day 3 + zygote	39,3%	1,78	26%
Scott et al., 2000	Day 3 + zygote	57%	3,2	31%
Van Royen et al., 1999	Day 3	63%	2	49%
Huisman et al., 2000	Day 5	27,8%	1,9	26%
Rijnders and Jansen 1998	Day 5	53%	2,25	30%
Coskun et al., 2000	Day 5	39%	2	24%
Shapiro et al., 2000	Day 5	63%	2,73	43%
Milki et al., 2000	Day 5	68%	2,4	47%
gardner et al.,2000	Day 5	87%	2	70%

embryos are transferred on day 3, whereas these rates drop severely if culture was continued for several days to obtain blastocysts for transfer. These findings have been confirmed in an American study (Racowsky et al., 2000). The second paper concerns a comparison of 48 day-3 transfers, where embryos were selected either on the basis of both pronuclear

criteria (according to Scott and Smith, 1998) *and* day-3 criteria, or solely on the basis of day 5 criteria (Rienzi et al., 2002). They concluded that there was no difference between the transfer of two day 3 versus two day 5 embryos; on the contrary, more embryos were available for freezing in the day 3 transfer group so that the authors concluded that day 3 transfer is in fact superior over day 5 transfer when expressed per oocyte retrieval, but at least one cryopreservation cycle is necessary to demonstrate this superiority. The third randomized trial also could not find a difference between day 3 and day 5 transfers, although the baseline efficacy in this study was much lower. So it seems, that irrespective of the baseline efficacy of a program, day 3 transfers are at least as successful as day 5 transfer if two embryos are transferred.

In conclusion, there is no definitive evidence to state that as a prerequisite for eSET, clinicians consider that transfers must or should be performed on day 5 after oocyte recovery, rather than on day 3 or day 1. Transfer of blastocysts can only be defended if it is shown and confirmed unambiguously, that this extra effort creates a significant net gain in OPR as compared to transfers at earlier stages. It is not impossible, although it remains to be demonstrated, that for the small group of patients who produce two or more top quality day 3 embryos, blastocyst culture may have an advantage.

**TABLE 19.9. PROPORTION OF TRANSFERS OF A SINGLE,
TWO, THREE OR MORE THAN THREE
EMBRYOS OVER A FOUR YEAR PERIOD**

	1998	1999	2000	2001	98-01
N OPU's	340	408	395	416	1559
N transfers (%)	317 (93)	388 (95)	370 (94)	389 (93)	1464
1ET (%)	42 (13)	100 (26)	122 (33)	121 (31)	385 (27)
1ET TOP (%)	31 (9.8)	75 (19.3)	101 (27.3)	92 (23.6)	299 (20.4)
2ET (%)	195 (62)	224 (58)	197 (53)	237 (61)	853 (58)
3ET (%)	55 (17)	47 (12)	39 (11)	24 (6)	165 (11)
>3ET (%)	25 (8)	17 (4)	12 (3)	7 (2)	61 (4)

THE PROOF OF THE PUDDING: WHAT CAN WE EXPECT FROM A GRADUAL INTRODUCTION OF ESET?

We describe our own experience as an example of what can be expected when eSET is gradually introduced into an ongoing IVF/ICSI program. At the start, in 1996, with the transfer of the two "best looking" embryos as the standard policy, a very high multiple pregnancy rate (40% of all ongoing pregnancies) compelled the clinicians to find a solution. Since the policy at that time was already to transfer no more than two embryos during the first two cycles, and since the large majority of multiples were twins, the only *logical* solution was to introduce single embryo transfer. The biologists were willing to cooperate if they were given time in order to try to actively select embryos on the basis of a validated system rather than on the basis of the rather vaguely described criteria currently found in the literature.

PHASE I: PRECLINICAL PHASE: DEFINING AND VALIDATING CHARACTERISTICS OF A TOP QUALITY EMBRYO.

During 1996, a consecutive series of 23 twins resulting in the birth of 46 healthy babies, the characteristics of 46 implanting embryos were analyzed. On the basis of this analysis, strict embryo criteria (cfr. supra: absence of multinucleated blastomeres, 4 or 5 blastomeres on day 2, a minimum of 7 cells on day 3 and a maximum of 20% anucleated fragments) were defined and reported (Van Royen et al., 1999). In 1997, the standard procedure still consisted of the transfer of the two embryos, defined according to traditional morphological criteria, i.e. the two "best looking" embryos, i.e. taking into consideration only fragmentation and cleavage speed and choosing the embryos with the least fragmentation and the highest cleavage speed but not looking at multinucleation. The whole IVF/ICSI population of that year was retrospectively analyzed (400 cycles of which 221 with double embryo transfer) in order to validate these "strict embryo criteria" (Table 19.3). The "top quality" or PHC embryo characteristics were validated in this second retrospective study as can be seen in Table 19.3 (Van Royen et al., 1999 (for the criteria); Gerris et al., 1999 (for the RCT comparing 1 with 2 top embryos).

PHASE II: CLINICAL TRIALS (ET-I AND SET-II STUDIES)

From 1998 onwards, two consecutive prospective studies were conducted: one prospectively randomized trial comparing the result of the transfer of one or two PHC embryos in women <34 years of age DURING their first IVF/ICSI cycle (SET-I study) (Gerris et al., 1999); and one non-randomized prospective impact study comparing the result of SET versus

DET (= double embryo transfer) in women <38 years of age in their first IVF/ICSI cycle; the second study was conducted both from the clinical point of view and from the health-economic point of view (SET-II study; in preparation).

SET-I STUDY (1998-1999)

The results of the SET-I study presented in Table 19.4 showed that a high ongoing pregnancy rate was possible after the transfer of one PHC embryo. In this study, patients were asked to participate in this *randomized* controlled trial comparing the transfer of one versus two top quality embryos; those who participated and who produced two top quality embryos were randomly allotted to one of both study arms. Given the strict inclusion criteria, this resulted in a small increase of the proportion of single embryo transfers to 10% of all transfers. This increase was limited because many patients did not participate and of those who agreed, only a fraction produced the two top quality embryos necessary for randomization. On the other hand, because the study counseling stressed the risks of twin pregnancy, the number of patients spontaneously requesting eSET also slightly increased during this period. The percentage of transfers of a single top quality embryo therefore slowly rose to 19% in 1999. During this first year elective SET was introduced in the program, there was no drop in the overall ongoing pregnancy rate of the program.

SET-II STUDY (2000-2001)

From January 2000 onwards, the SET-II study was conducted. This was a multicenter long-term health-economic study with follow-up of all children until three months after delivery. Its primary endpoint was a comparison between the total costs of twin versus singleton pregnancies after IVF/ICSI. In this study, all patients <38 years of age and undergoing their first IVF/ICSI treatment or a first IVF/ICSI treatment after a previous delivery were allowed to *choose* either a double transfer (DET) of the two best embryos (i.e. either two PHC embryos, one PHC and one non-PHC embryo, or two non-PHC embryos) or a single embryo (SET). The same characteristics were used to define a PHC embryo as in the previous studies. They received SET if a PHC embryo was available, and if not, a DET of the two best embryos. For clarity: the agreement (= principle) we made with the patient was that she could choose SET or DET, but SET automatically meant that she would have to had at least one PHC embryo. However, some patients who did not produce a PHC embryo nevertheless wanted only one embryo at any cost. That is in part how the group SET of a non-PHC embryo was formed, the other way being patients with compulsory a SET of a non-PHC embryo. This

resulted in a substantial increase in the number of single embryo transfers, since 66% of eligible patients in our center chose SET and 34% chose DET. In 2000, 26% of all transfers were transfers of a single top quality embryo, i.e. about one hundred SETs during that year.

In the course of these two studies, the original strict embryo criteria were further fine-tuned using a new model to calculate the implantation potential of day three embryos in women <38 years of age, in which it was again shown that embryos with multinucleated blastomeres (MNB) have a very poor implantation potential (Van Royen et al., 2000; Table 19.14). Given the high incidence of MNBs (>40% of all embryos), the importance of this criterion should not be underestimated. These “fine-tuned” criteria further confirmed the validity of the original “strict” criteria (= *absence of multinucleated blastomeres, 4 or 5 blastomeres on day 2, a minimum of 7 cells on day 3 and a maximum of 20% anucleated fragments*) but it was noticed that some groups of embryos who were not “covered” by the original description of a PHC embryo also appeared to have a very high implantation potential. In Table 19.14, the groups of embryos “covered” by the original “strict” criteria are indicated in bold. It is shown that in fact, looking at cell cleavage speed (some embryos catching up), fragmentation and absence of multinucleation (all embryos in Table 19.14) can make a ranking of implantation potential of embryos. So the main difference with traditional criteria consists in excluding embryos with MNB’s (multinucleated blastomeres) even if they look “perfect” with respect to cleavage speed and percentage of fragmentation.

To a lesser extent, it means that the quickest cleaving embryos are not necessarily PHC embryos, because apparently embryos may cleave not only too slowly but also too quickly, whereas some embryos that start off rather slowly (2 cells on day 2) could catch up; and thirdly, fragmentation appeared not to be the most important thing to look at as long as it remains <20%.

In summary, over a four year period SET was applied in a gradual manner in three categories of patients: 1. women participating in the SET-I study; 2. women participating in the SET-II study and 3. women who did not fulfil the inclusion criteria of either study but nevertheless wanted only one embryo replaced, including, some patients >38 years of age. The overall results of the two studies as well as in women who did not (i.e., want to) participate in either study are shown in Tables 19.11 and 19.12. It is essential to state that no patients were compelled to receive only one embryo – in all cases, they could elect for two embryo transfers (in the SET-I study period by not wanting to participate to the study; in the SET-II study by making a direct choice). Moreover, patients allotted by randomization (SET-I) or by their own choice (SET-II) to have one embryo transferred, received only one embryo on the condition that it was a PHC embryo, as strictly defined. Moreover some women received one

non-PHC embryo at their own request (usually women with children) but the majority selected to have two best non-PHC embryos replaced.

For clarity's sake, I summarize the composition of the patient material, which may seem somewhat confusing but which reflects the reality of IVF when applying SET and using the notion of PHC embryo. In SET-I (1998-1999) the patients *could not choose*, it was a RCT; only patients with at least 2 PHC could be entered in the comparison. Of course they had the choice not to participate in the study; those who did not want to participate usually requested a two embryo transfer but some did choose a one (PHC or not) embryo.

In SET-II (2000-2001) patients *had to choose*: 1 (only if a PHC embryo) or 2 (either 2 PHC embryos, 1 PHC embryo or no PHC embryo); but: even if there was no PHC embryo available, some patients who had chosen SET at the time of counseling under the presumption that they would have a PHC embryo available, still wanted only 1 embryo at the time of transfer (especially women with children already), that is part of the non-top SET group, the other part being patients who had only one embryo of non-PHC. On top of SET-I and SET-II there were patients who *demanded SET*, irrespective of the fact whether the embryo was a PHC embryo or not. All these groups are recognizable as such in the Tables.

PHASE III: PRESENT EVALUATION OF OVERALL EXPERIENCE WITH ESET

We studied an uninterrupted series of 1559 IVF/ICSI cycles over a period of four years (1998-2001). Patients were treated for all accepted indications for IVF/ICSI, including microscopic epididymal sperm aspiration (MESA) and testicular sperm extraction (TESE) in cases of obstructive and non-obstructive azoospermia, respectively. Mean age of the female partner was 32.4 ± 4.4 years. Treatment cycle rank varied between 1 ($n=820$) and 13 ($n=1$). Of a total of 1559 ovum retrievals, 1464 transfers resulted, of which 385 were a single embryo (26.3%): 299 (20.4%) were a single PHC embryo, and 85 (5.9%) were a single non-top quality embryo. This report (Gerris et 2002) considers the complete population of patients who received a single embryo and describes the results in these patients compared to all other patients receiving two or more embryos. It should indeed be underlined that, during the whole period described, there were of course still transfers of three embryos, e.g. in the >38 years group, or in women in their third and fourth trial. The gradual impact of the progressive introduction of eSET over these four years on the overall ongoing pregnancy rate and the multiple pregnancy rate (per year) was analysed. Materials and methods are described elsewhere (Gerris et al., 2000).

TABLE 19.9. PROPORTION OF TRANSFERS OF A SINGLE, TWO, THREE OR MORE THAN THREE EMBRYOS OVER A FOUR-YEAR PERIOD

	1998	1999	2000	2001	98-01
N OPU's	340	408	395	416	1559
N transfers (%)	317 (93)	388 (95)	370 (94)	389 (93)	1464
1ET (%)	42 (13)	100 (26)	122 (33)	121 (31)	385 (27)
1ET TOP (%)	31 (9.8)	75 (19.3)	101 (27.3)	92 (23.6)	299 (20.4)
2ET (%)	195 (62)	224 (58)	197 (53)	237 (61)	853 (58)
3ET (%)	55 (17)	47 (12)	39 (11)	24 (6)	165 (11)
>3ET (%)	25 (8)	17 (4)	12 (3)	7 (2)	61 (4)

TABLE 19.10. PRECENTAGE OF SINGLE EMBRYO TRANSFERS, MEAN NUMBER OF EMBRYOS TRANSFERRED, ONGOING IMPLANTATION RATE (OIR), ONGOING PREGNANCY RATE (OPR), TWINNING RATE AND TOTAL MULTIPLE PREGNANCY RATE IN AN IVF/ICSI PROGRAM WHERE ESET WAS INTRODUCED OVER A FOUR YEAR PERIOD

All cycles (n= 1559)	1998	1999	2000	2001	98-01
% SET	13	26	33	31	27
N embr/ET	2.26	1.96	1.85	1.79	1.95
OIR (%)	23.5	20.1	22.7	22.4	22.1
OPR/OPU (%)	35.9	27.9	31.9	31.0	31.5
OPU/ET (%)	38.5	29.4	34.1	33.2	33.5
Twins (%)	29.5	30.7	20.6	16.3	24.0
Tot. Mult.(%)	33.6	32.5	22.2	18.6	26.5

Table 19.9 shows the proportion of transfers of a single, two, three, and more than three embryos in a total series of 1559 IVF/ICSI cycles over this four years' period (1998-2001). Table 19.10 illustrates the evolution of the proportion of single embryo transfers, the mean number of embryos transferred, the ongoing pregnancy rate, the ongoing implantation rate, the twin pregnancy rate and the total multiple pregnancy rate over these years during which single embryo transfers were gradually applied. The total multiple pregnancy rate dropped from 33.6% in 1998 to 18.6% in 2001. The incidence of twins over the same period dropped from 29.5% in 1998 to 16.3% in 2001. The progressive effect of an increasing proportion of eSET over the years is reflected in a steady decline in the average number of embryos transferred and a parallel decrease in the multiple pregnancy rate (Fig. 19.1). The overall ongoing pregnancy rate remained stable; an average of 2.26 embryos per transfer in 1998 resulted in 35.9% ongoing pregnancies per oocyte retrieval; an average of 1.85 embryos per transfer in 2000 resulted in 31.9% ongoing pregnancies per retrieval; an average of 1.79 embryos per transfer resulted in 31.0% ongoing pregnancies per retrieval in 2001.

TABLE 19.11. OUTCOME OF THE TRANSFER OF A SINGLE TOP QUALITY EMBRYO (N=299), A SINGLE NON-TOP QUALITY EMBRYO (N=86), TWO EMBRYOS (N=853) OR MORE THAN TWO EMBRYOS (N=226) IN AN IVF/ICSI PROGRAM (ALL AGES)

All ages	SET TOP (%)	SET non TOP (%)	all DET (%)	>2 ET (%)	all non SET (%)	TOTAL (%)
n transfers	299 (20.4)	86 (5.9)	853 (58.3)	226 (15.4)	1079 (73.7)	1464
N conceptions	149 (49.8)	26 (30.2)	424 (49.7)	93 (41.2)	517 (47.9)	692 (47.3)
n ongoing pregnancies	105 (35.1)	19 (22.1)	309 (36.2)	57 (25.2)	366 (33.9)	490 (33.5)
n singlettons	104 (99)	19 (100)	200 (64.7)	37 (64.0)	237 (64.7)	360 (73.5)
n twins + n triplets	1 (1)	0	104 + 5 (35.3)	13 + 7 (37.7)	117 + 12 (35.2)	130 (26.5)

In conclusion, there was a constantly high ongoing implantation rate, due to better embryo selection. This resulted in a stable high ongoing pregnancy rate per oocyte, although the number of transferred embryos

decreased, and a steadily declining multiple pregnancy rate was accomplished (Fig.19.2). Table 19.11 shows the clinical outcome variables of 385 single embryo transfers compared with 1079 transfers of two ($n=853$) or more than two ($n=226$) embryos in all age groups. There were 299 transfers of one top quality embryo, as previously defined (Van Royen et al., 1999, 2001): absence of multinucleated blastomeres, 4 or 5 blastomeres on day 2, a minimum of 7 cells on day 3 and a maximum of 20% anucleated fragments. These resulted in 149 implantations (49.8% (= hatched blastocysts); this means: in clinical terms, biochemical pregnancies, i.e. at least two subsequently rising hCG values. In biological terms, at least one out of two transferred embryos became a blastocyst that moreover hatched since it produced hCG; probably even a higher percentage of blastocyst formation occurred since blastocysts may form that do not even begin implantation; this suggests between the lines that a day 3 PHC embryo selected by the criteria we used is doing as well as the best published results for day 5 transfers. There were 105 ongoing pregnancies (35.1%) (including one monozygotic twin); 16 clinical miscarriages; three ectopic pregnancies; and 25 biochemical conceptions. The other 86 transfers of a single non-top quality embryo resulted in 26 conceptions (30.2%), of which five were biochemical, two were clinical miscarriages and 19 were ongoing singletons (22.1%). In the other 1079 embryo transfers, performed in second or higher rank cycles and in patients over 38 years of age, and who did not demand of their own will to have a single embryo transfer, an average of 2.3 embryos were transferred, resulting in a total of 516 conceptions (47.8%), of which 366 were ongoing pregnancies (33.9%). In the SET group, there was one monozygotic twin pregnancy, whereas the non-SET transfers resulted in 117 twins (32%) and 12 triplets (3.3%), of which 5 were dizygotic (after transfer of two embryos only). Table 19.12 shows the outcome of the 853 double embryo transfers separately, broken down into three groups: two, one and no top quality embryos. The ongoing implantation rate was 35.1% for (top quality) embryos transferred in the eSET group and 36.5% in the DET group that received two top quality embryos. For the DET group with one top and one non-top quality embryo, the ongoing implantation rate was still high (27.2%), whereas in the two non-top quality embryo DET group, it was only 11.8%. In case a conception occurred, the odds for a biochemical conception were significantly higher in the no top quality DET group than in the two top quality DET group ($OR=1.12$; 95% CI=1.00–1.26). The odds for a multiple pregnancy (twins and dizygotic triplets combined) was significantly lower when no top quality embryos were transferred in the DET group as compared with both the two top quality DET group ($OR=0.677$; 95% CI=0.568–0.807) and with the one top plus one non-top quality embryo DET group ($OR=0.802$; 95% CI=0.666–0.967) (Table 19.13). Dizygotic triplets ($n=5$) occurred

only in patients who received two top quality embryos, four in women <38 years of age and one in a woman of 39 years. The seven other triplets occurred in patients who received three embryos because they were in a high rank IVF/ICSI trial (i.e. in their fourth or higher rank trial).

TABLE 19.12. CLINICAL OUTCOME VARIABLES IN PATIENTS RECEIVING ONE (SET) OR TWO (DET) EMBRYOS, AS A FUNCTION OF THE NUMBER OF TOP QUALITY EMBRYOS (ALL AGES)

All Ages	SET TOP (%)	SET non TOP (%)	DET 2 TOP (%)	DET 1 TOP (%)	DET 0 TOP (%)
N transfers	299	86	322	209	322
N conceptions (A)	147 (49.8)	26 (30.2)	207 (64.3)	117 (56)	99 (30.7)
N ongoing pregn. (B)	105 (35.1)	19 (22.1)	160 (49.7)	84 (40.2)	65 (20.2)
N bioch pregn.	25 (17)(of A)	5 (19.2)	26 (12.6) (of A)	22 (18.8)	22 (22.2)
N miscarriages	16 (10.9)	2 (2.3)	17 (8.2)	10 (8.5)	10 (10.1)
N ectopics	3 (2)	0	4 (1.9)	1 (0.8)	2 (2)
N singletons	104 (99)(of B)	19 (100)	90 (56.3) (of B)	56 (66.7)	54 (83.1)
N twins	1	0	65 (40.6)	28 (33.3)	11 (16.9)
N dizygotic triplets	0	0	5 (3.1)	0	0
Ongoing implant rate	105/299 = 35.1%	19/86 = 22.1%	235/644 = 36.5%	112/418 = 26.8%	76/644 = 11.8%

REFLECTIONS REGARDING THE INTRODUCTION OF ESET IN AN ONGOING IVF/ICSI PROGRAM

ESSENTIAL RECOMMENDATION

Notwithstanding a tremendous amount of compelling evidence for the serious complications of multiple pregnancy, the actual high incidence of multiple pregnancies after treatment with assisted reproductive technologies remains largely unchanged. Apart from many efforts to analyze which clinical and embryological factors correlate with the risk for multiple pregnancy, prevention until now has been focused largely on the prevention of high-order multiple pregnancies (i.e. triplets and more), leaving twins unaffected. It is clear, however, that reducing the number of transferred embryos from three to two in the general IVF population results in a sharp decrease of the incidence of triplets without affecting the overall (ongoing) pregnancy rate (Staessen et al., 1993, Templeton et al., 1998). A further linear reduction from two embryos to one embryo in the general population would certainly result in a severe drop of the total (ongoing) pregnancy rate and would therefore in fact unacceptable for the patients. It has been suggested however (Coetsier and Dhont, 1997; Strandell et al., 2000) that a substantial proportion of twins could easily be avoided without much of a drop in overall pregnancy rate.

CALCULATING THE POTENTIAL EFFECT OF ESET

The most recent data, including the findings reported here, indicate that a decrease in the number of embryos transferred from two to one is a reasonable option in at least one third of the population of patients, thereby reducing the twin incidence to approximately half of its original incidence, without causing any decline in the overall ongoing pregnancy rate of the program. The group receiving one top quality embryo had the same overall ongoing pregnancy rate as the group receiving more than one embryo, supporting our criteria for both embryo (absence of multinucleated blastomeres, 4 or 5 blastomeres on day 2, a minimum of 7 cells on day 3 and a maximum of 20% anucleated fragments) and patient selection (<38 years of age, first IVF/ICSI cycle).

Our present policy (from Jan 1st 2002 onwards) consists of giving one embryo in all first IVF/ICSI cycles where a top quality embryo is obtained, in as many second IVF/ICSI cycles as possible where a top quality embryo is obtained and even in third or higher rank IVF/ICSI cycles where a PHC embryo is obtained and if the patient agrees. In all other cycles, two (non-PHC embryos) are transferred. The latter predictably results in an acceptable ongoing pregnancy rate (20%) with an

“acceptable” twinning rate of 16% in this group (Table 19.12). I think it is “acceptable” because it is approximately half of what it used to be.

Counselling is very important to make this procedure work. The transfer policy should be fully discussed with the couple well before the transfer (even before the stimulation starts). The biologists or the transferring doctor are under no circumstances to be put under pressure to change what had been agreed upon; transfer is an emotionally laden moment and hardly the moment to make a balanced decision, so we have to agree beforehand and then stick to that decision.

How twins are “viewed” by the patient is important, not as a basis for decision-making, but as a basis for counselling. Medicine should not be based on strong opinion but on documented facts. Of course, if the doctor is not convinced of the statistical fact that twins create more problems than singletons (because he or she never sees them) or because he is afraid his figures will be somewhat down, he or she will not sound convincing to the patient.

It is interesting to calculate from the figures in Table 19.12 the potential total effect of single embryo transfer on this program: in the group of 853 double transfers, all patients who produced at least one top quality embryo ($n = 322 + 209 = 531$) would have received only one embryo. Assuming the same ongoing implantation rate as for single transfers of a top quality embryo (35.1%), then $531 \times 35.1\% = 186$ ongoing pregnancies would have been obtained, instead of $160 + 84 = 244$; i.e. 58 fewer pregnancies, but all would have been singletons. Again, from the patients perspective, they might have benefited from DET, but we think it is the better clinical practice to “sacrifice” better embryo selection on the altar of twin prevention, because our overall OPR remained stable. As suggested before, the bottom-line is a moral-ethical one. There would have been only eleven twins (those occurring in the two non-top quality embryo DET group) in a total of $186 + 65 = 251$ ongoing pregnancies, i.e. 4.4% twins, instead of what we actually obtained: 109 twins (including 5 dizygotic triplets) in 309 ongoing pregnancies (35.3%). In other words: we would have had $109 - 11 = 98$ fewer twins (-90%) for a mere $309 - 251 = 58 = 58/309 = 18.8\%$ fewer pregnancies, not taking into account a potential positive effect of cryopreservation (Tiitinen et al., 2000; Martikainen et al., 2000; Rienzi et al., 2002). This “loss” of 18.8% pregnancies should not be interpreted as a decrease that is to be expected if eSET is adopted in a program, but rather as the size of a potential gain in pregnancy rate that is voluntarily missed by not transferring two embryos if at least one top quality embryo is available. Therefore, if transfer of a single top quality embryo were to be made the standard of care (and not, as in this series, a possibility offered in the context of clinical trials), the overall effect on the twin pregnancy rate could still be

higher. This is illustrated by the gradual and progressive effect of an increasing proportion of eSET in this program.

The group of patients suitable for single embryo transfer may be smaller in other programs with patients who on average are older. It could also be larger if higher-rank treatment cycles with top quality embryos are also included. The first and foremost suitable groups of patients are young women in their first IVF/ICSI cycle. Combining validated strict developmental criteria of early cleaving embryos with a clinical profile of the twin prone patient, as described here and by others (Standell et al., 2000) results in an acceptably high and stable ongoing pregnancy rate of ~33.5% per started cycle (Table 19.10). This allows the transfer of a single top quality embryo to be considered as the standard of care in these patients. The application of transfer of a single top quality embryo may be extended to second and third treatment cycles, or perhaps to all cycles with at least one top quality embryo. A patient who did not produce a top quality embryo during her first cycle and who did not conceive an ongoing pregnancy after the transfer of two non-top quality embryos, but who does produce a top quality embryo in her second cycle, should probably also be a good candidate for eSET during that second cycle. The feasibility of extending single embryo transfer in a maximum number of cycles is also linked to the performance of the cryopreservation program. The role of cryopreservation is likely to become increasingly important as eSET finds its way into the clinic, as recently demonstrated by others (Martikainen et al., 2000; Tiitinen et al., 2000; Strandell et al., 2000).

TABLE 19. 13. ODDS FOR A BIOCHEMICAL PREGNANCY AND FOR A MULTIPLE PREGNANCY BETWEEN THE THREE SUBGROUPS OF DOUBLE EMBRYO TRANSFERS

	DET 2 TOP	%	DET 1 TOP	%	DET 0 TOP	%
Bioch/conception	26/207	12.6	22/117	18.8	22/99	22.2 ^a
Mult/ongoing pregn.	70/160	43.8	28/84	33.3	11/65	16.9 ^{b,c}

^a OR = 1.12 (95% CI=1.00-1.26) BETWEEN 2 TOP DET VS. NO TOP DET; ^b AND ^c OR = 0.677 (95% CI=0.568-0.807) AND OR = 0.802 (95% CI=0.666-0.967) FOR 2 TOP DET VS. NO TOP DET AND 1 TOP DET VS. NO TOP DET, RESPECTIVELY.

A GRADUAL APPROACH TO ESET IS RECOMMENDED

In order to avoid a decrease in the overall ongoing pregnancy rate of a program, we suggest that the introduction of single top quality embryo

transfer should be gradual and implemented in distinct clinical phases. First, each center should decide on how to select the high implantation embryo. Some centers may prefer extended culture to blastocysts (Gardner et al., 1998, 2000); others may (have to) focus on day 1 embryos (Scott and Smith, 1998; Ludwig et al., 2000); but most will keep to the current and successful day 2 or day 3 schedule (Figures 19.1 and 19.2). Local customs or regulations, legal constraints (e.g. Germany), as well as insurance policies, may play a disproportionately important role in this decision process. I prefer to transfer day 3 embryos because a prospectively randomized trial comparing the transfer of two day 3 embryos with two blastocysts did not show blastocyst culture to be superior in an unselected population (Coskun et al., 2000; Rienzi et al., 2002; Utsunomiya et al., 2002). In addition, we have obtained a conception rate of 64.3% (207/322) and an ongoing pregnancy rate of 49.7% (160/322) after transfer of two top quality day 3 embryos (Table 19.10), which is similar to published results for the transfer of two blastocysts. I believe it is important to adhere to strict embryo criteria, especially with respect to the presence of multinucleation, because these embryos are often aneuploid. In our center, the incidence of observed multinucleation (i.e. as little as just one blastomere showing multinucleation during one single observation has risen from 17% in 1998 to 34% in 2001; even if these embryos have little fragmentation and cleave according to the "optimal" pattern, they are not transferred. These authors also suggest that many programs do not use day 3 criteria optimally, resulting in suboptimal implantation rates. This gives blastocyst culture an apparent advantage, which disappears with the ability to *actively* predict which embryos have the highest chance to implant when assessments are made on day 3.

In parallel with the decision to use an embryo selection strategy, the center should undertake judicious patient selection. As confidence in the method comes with experience, eSET can then be proposed more liberally, possibly but not necessarily, resulting in some decline in the overall results, as illustrated by our experience. The application of eSET will, and probably should, always remain a matter of sound clinical judgment and common sense rather than of compelling mathematics. The idea of eSET is both simple in design and it is a low-tech procedure. To put it in practice one needs only to be convinced of the high risk of twin pregnancy. A change in mentality is mandatory, not least among reproductive physicians and their collaborators, i.e. all those working in the IVF unit. It can only be hoped that eSET will gradually become the standard of good clinical practice. Financial arguments are futile, as costs for twins are in the long run much higher than for singletons, both for parents and for health-care insurers, whether public or private. Studies show that, if properly counseled, patients readily accept eSET of a PHC

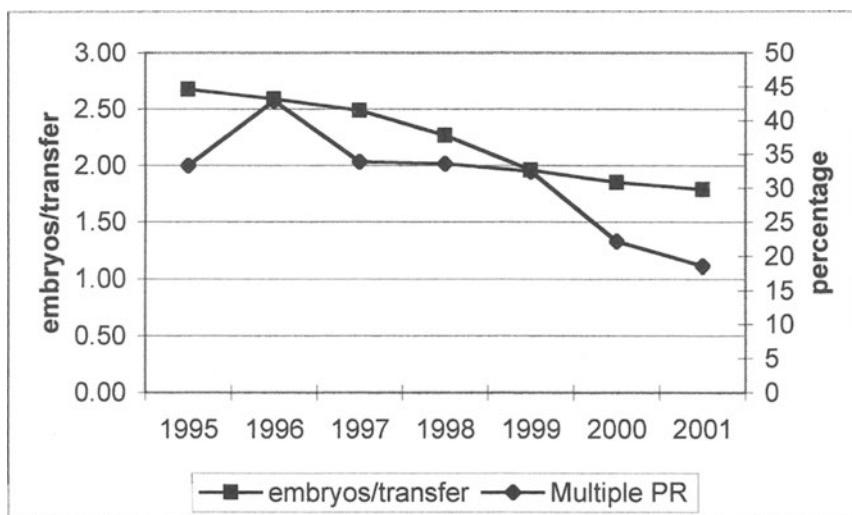


FIGURE 19.1. Evolution of the total multiple pregnancy rate and of the mean number of day 3 embryos transferred with gradual introduction of elective single embryo transfer in an IVF/ICSI program.

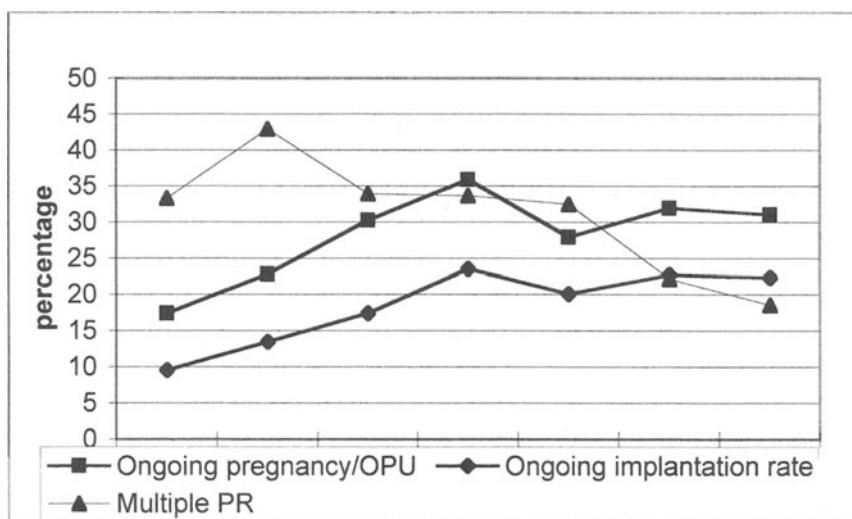


FIGURE 19.2. Evolution of the ongoing pregnancy rate, the ongoing implantation rate and the multiple pregnancy rate in an ongoing IVF/ICSI program where eSET was gradually introduced.

embryo during their first IVF/ICSI cycle (De Neubourg et al., 2002). Moreover, it has been shown using a decision-analysis approach that the cost per child after SET and after DET are comparable, whatever the baseline efficacy of the program, but the real difference in medical and other costs start after birth (De Sutter et al., 2002). The main risk to be avoided is an undiscriminating legislation that would make SET, as opposed to elective SET, compulsory, irrespective of strict embryo quality considerations or irrespective of the clinical profile of the patient.

THE TOP QUALITY EMBRYO: IS IT TOP QUALITY FROM THE BEGINNING OR DOES IT BECOME TOP QUALITY DURING ITS DEVELOPMENT?

The intrinsic implantation potential of an embryo can never be increased without intervention, but only be preserved as much as possible during culture *in vitro*. Pure reason says that all embryos that implant must necessarily pass through the blastocyst stage, which is the most developed stage at which embryo transfers are presently conducted. The challenge of optimal embryo selection therefore can be solved only in two ways. The first is to adopt an anticipatory attitude consisting of culturing embryos to the blastocyst stage and transferring *the* single blastocyst that is supposed to have the highest implantation potential. The second is to try to actively identify, at an earlier stage, the *same* embryo(s) with the (same) high implantation potential *before* it becomes a blastocyst. Even when excluding the very likely possibility for any embryo the fact of being in an extracorporeal environment may only cause loss but never a gain of intrinsic implantation potential, the alleged difference that might be (but as yet has not yet been) demonstrated between the efficiency of identifying the PHC embryo on day 3 compared with day 5 stage, does not reside in an *intrinsic* difference between the different stages of development of the *same* embryo, but rather between the ability of optimal identification at a particular stage. The same reasoning is valid when comparing day 3 embryos with day 2 embryos or with embryos at the pronuclear stage. The fact that an embryo develops to the blastocyst stage is certainly no proof of its normalcy, since aneuploidies have been demonstrated in blastocysts (Evsikov and Verlinsky, 1998).

Papers that show a higher ongoing implantation rate for blastocysts than for day 3 embryos invariably show implantation rates for day 3 embryos that are (far) below what is obtained in centers that have adopted a validated early stage embryo selection procedure. The difference between both seems to be an indicator for embryo selection at day 3 in a particular center rather than of an intrinsic difference between day 3 and day 5 embryos. This is not to say that blastocyst culture, in patients who happen to have two or more top quality embryos on day 3 (which is a

small minority), may have an additional potential for further selection. But this is a relatively minor issue compared with the major issue of avoiding twins in the twin prone patient group, which comprises the majority of women <38 years in their first two IVF/ICSI cycles. The debate concerning avoiding twins in the young first cycle patient is very different from the debate regarding the difficult or older patient in whom high implantation embryos are not or rarely obtained. Differences shown in Table 19.8 should be viewed from this point of view.

One may question the use of morphological and growth characteristics of embryos as a fundamental selection tool. When using embryo morphology, it is only assumed, but not proven, that an embryo with a normal genetic constitution is likely to be an embryo with an ideal cleavage speed, low incidence of fragmentation, symmetrical blastomeres, absence of multinucleation, etc. But morphology does seem to be a useful tool in the present circumstances. However, genetic defects can and do interfere with postimplantation and fetal development, and morphology during the preimplantation stages is an unreliable indicator of certain genetic and chromosomal defects that result in a viable pregnancy. The ideal identification technique, until now theoretical, would be to perform a complete assessment of the embryo's genetic constitution; i.e. it's nuclear genome and its mitochondrial DNA. This approach would probably be near perfect, but expensive, invasive and probably induce genetic risks and embryo loss due to the manipulation. This approach also assumes that epigenetic or nongenetic factors play a minor role in the establishment of competence, a notion that may in fact be false.

Since we are far from this possibility, the second best approach is to observe a limited number of easily recordable embryo characteristics and correlate them with a documented implantation potential. This approach is non-invasive and allows quick application in the clinic. It can be done only with embryos with a 100% certain outcome, i.e. for transfers followed by a positive HCG and a "one-to-one" transfer, i.e. ongoing singletons after SET, twins after DET and triplets after triple embryo transfers. Embryo performance in vitro would thus include growth kinetics and morphology, which may then be considered as a correlate of the presumed genetic constitution of the embryo. This correlation is necessarily a relatively poor one, given the many stages at which development can be arrested. It is therefore reasonable to think that a technique which is able to predict an implantation potential of ~50% (as in the patients described here who received one top quality day 3 embryo) is almost at the limit of its possibilities. It is possible that incorporating embryo characteristics from earlier or later stages may either add or even replace the selection potential proposed by us for day 2 and day 3. However, looking at the number and the distribution of nucleoli on day 1 is certainly not easier to perform than identifying multinucleated blastomeres.

Culturing embryos until day 5 is equally not any easier or cheaper than transferring embryos on day 3. Of the characteristics we are currently using, identification of multinucleated blastomeres is probably the most influential one. Multinucleated embryos may have an ideal morphology in terms of number of blastomeres and limited fragmentation, and still have a very poor implantation potential (Pickering et al., 1995; Pelinck et al., 1998; Jackson et al., 1998). Fragmentation is probably not of the same importance. It can be seen as a physiological mechanism of equilibrating the volume of the cell's cytoplasm with its nuclear activity, whereas the number and symmetry of the blastomeres is probably such a primary and fundamental characteristic of normal cleavage that it identifies nothing more than the large cohort of early cleaving embryos in which further selection has to take place. In our overall experience, 82% of all ongoing implantations were the result of top quality embryos and only 18% of non-top quality embryos. At present, the assessment of day 3 embryos in many programs is probably suboptimal. Ideally, all embryos should be systematically examined at fixed times during each day of development.

Day "2" and "day 3" represent a full 24-hour interval. Looking at an embryo in one case 26 and in another case 36 hours after fertilization (both observations are made on day 2) may yield quite different observations. All embryos should be evaluated at fixed and strictly applied time intervals. For each embryo a fixed number of variables must be systematically recorded, so that a constant monitoring of the ongoing implantation rate of a program, as a function of these variables, can be performed. There must be full adherence to strict working protocols by all the members of the laboratory team. There must be a systematic recording of the clinical outcome of all the embryos.

When applying this method, we were able to detect at least one PHC embryo in ~2/3 of all first and second IVF/ICSI cycles. Initially this figure was 75% but with a better identification of multinucleated embryos (17% in 1998, 37% in 2001), apparent top quality embryo formation rate dropped somewhat. Transfer of such embryos resulted in ~50% implanting blastocysts (leading to at least a biochemical pregnancy or beyond) and ~35% of ongoing implantations. We have yet to see published data, in an otherwise unselected population of patients in their first or second IVF/ICSI cycle, which shows higher figures for blastocyst transfer. Reported ongoing pregnancy and multiple pregnancy rates after the transfer of two blastocysts, usually in patients selected by a high number (>10) of follicles or by the presence of at least three 8-cell embryos on day 3, are not higher than what we observe after the transfer of two top quality embryos with our day 3 scoring system (Table 19.12), i.e. 64%, with 41% of twins! Moreover, studies that report high pregnancy rates after blastocyst transfer often omit to mention the frequency of

patients not reaching embryo transfer because no transferable blastocysts were obtained.

Clearly, the final answer can only come from a prospective randomized study comparing the transfer of one day 3 embryo with one day 5 blastocyst. However, such a study should comply with the following methodological prerequisites: randomization must take place before the cycle is initiated; patients randomized for day 3 transfer receive the embryo considered to be the best using strict assessment criteria including multinucleation; the overall incidence of multinucleation of the program should be ~35-40% of all embryos; patients randomized for transfer on day 5 who do not produce a blastocyst must be included in the analysis; the ongoing implantation rate for day 3 embryos must be ~35% in order to prove optimal selection on day 3. The null hypothesis is that there will be no difference between both groups. Patients who have more than one PHC day 3 embryo should be analyzed as a separate group in order to assess whether in this group, blastocyst culture and transfer might add to the efficiency of earlier stage selection. The study should preferably be repeated in, e.g., five centers.

Finally, it is disquieting to see that a bulky literature exists on sperm morphology, including very sophisticated methods of computer-assisted evaluation. Workshops, symposia and congresses are dedicated to this admittedly important topic, whereas embryo morphology has not been the theme of such intensive endeavours. Sperm morphology is about correlations between *in vivo* fertilizing potential of a huge population of cells, produced for examination, where anything may happen with respect to the ejaculate that is deposited in the vagina at another moment in time. Embryo morphology is about a few clusters of cells that are the very ones that are going to be used in the hope to obtain a pregnancy. There is a great need for objective learning and teaching, for a systematic search for those characteristics that will help us to select the right embryo to transfer.

HOW TO COUNSEL PATIENTS REGARDING ESET?

It goes without saying that all counselling must comprise a transparent and detailed description of the risks and complications of multiple pregnancy, ideally accompanied by a short visit to a neonatal department. No counselling can be effective if the physician himself is not convinced of these risks. The good outcome of many twin pregnancies does not testify to the absence of risk. These risks should be presented in the context of reality, probability of complications and, to a lesser extent, patient desires. Twins have more prematurity (x7), cerebral palsy (x4), congenital abnormalities (x2-3) and neonatal mortality (x5). The financial dimension should be explained, stating that the extra burden begins after birth. The extra cycles necessary with eSET to obtain the same number of

children after DET do not cost more than the extra cost related to twin pregnancy and delivery: decision analysis has shown that both are comparable. The difference starts at birth. Some complications, such as neonatal death, cost nothing in terms of money but weigh a whole life on a person's soul. Overt morbidity, retarded development, neurolinguistic and emotional problems generate costly management, often for many years. With respect to the outcome of a particular IVF/ICSI cycle, the most useful tool would be a clear table of the type shown in Table 19.12 where the patients can see for themselves what is to be expected after the transfer of one or two embryos. Similarly, once enough observations are recorded for embryos with a certified 1:1 outcome, patients can also see for themselves what is to be expected (Table 19.14). In principle, the implantation of one embryo is independent of that of another embryo. For each combination of two embryos, and within a certain interval of confidence, the percentage of no conception, of singleton pregnancy and of twin pregnancy can be calculated. This has been done in a theoretical model (Martin and Welch, 1998), but it can be applied with real figures as well, on the proven assumption that the implanted fraction can be predicted for a particular embryo.

TABLE 19.14. OBSERVED AVERAGE RATES FOR EMBRYOS WITHOUT MULTINUCLEATION IN FUNCTION OF THE NUMBER OF BLASTOMERES AND THE DEGREE OF FRAGMENTATION ON DAY 2 AND 3.

Embryo type	Implanted fraction
<u>4-8 F1</u>	<u>42%</u>
<u>4-9 F2</u>	<u>42%</u>
<u>4-9 F1</u>	<u>37%</u>
<u>4-8 F2</u>	<u>36%</u>
2-8 F1	33%
<u>5-8 F1</u>	<u>30%</u>
<u>5-9 F1</u>	<u>30%</u>
2-7 F1	27%
<u>5-10 F1</u>	<u>27%</u>
6-10 F1	27%
<u>5-10 F2</u>	<u>27%</u>
<u>5-7 F2</u>	<u>25%</u>
<u>4-7 F1</u>	<u>23%</u>
<u>5-9 F2</u>	<u>23%</u>
<u>4-7 F2</u>	<u>22%</u>
<u>5-8 F2</u>	<u>21%</u>
<u>5-7 F1</u>	<u>19%</u>
2-6 F1	17%
4-4 F2	17%
4-6 F2	17%
<u>4-10 F1</u>	<u>16%</u>
2-6 F2	15%
3-8 F1	13%
4-6 F1	10%
3-5 F2	10%
3-6 F2	8%
5-5 F1	6%
4-5 F2	6%
2-4 F1	4%
2-4 F2	4%
4-4 F1	0%

TOP QUALITY EMBRYOS ARE UNDERLINED AND CORRESPOND TO THE DESCRIPTION BY VAN ROYEN ET AL (1999)

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CHAPTER TWENTY

CAN AND SHOULD HUMAN EMBRYOS BE “RESCUED” FROM DEVELOPMENTAL DEMISE?

METHODS AND BIOLOGICAL BASIS

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INTRODUCTION

The notion of “rescuing” human oocytes or embryos that are presumed to be dysfunctional owing to morphology or performance in vitro raises fundamental questions of what is “normal” and whether “rescue” is necessarily in the best “interests” of the entity being rescued. Before I address this, let us discuss the meaning of “normality” as we have problems deciding what this is for adults, let alone for embryos. Here in Australia, the New South Wales Supreme Court was recently asked to decide on whether a disabled child can sue its doctor for being born: the so-called “wrongful life” principle. Similar cases have been dismissed in the UK and France but others have succeeded in the USA. The three particular cases here concerned a young woman born blind, deaf, mentally retarded and in need of constant care after her mother contracted rubella in pregnancy; a boy born following a failed vasectomy who has intellectual disability, speech impairment and facial disfigurement, and a boy born with an inherited blood-clotting disease conceived by in vitro fertilization (IVF). In the last case the claim is that suitable genetic screening could have prevented the embryo from being transferred, even though it is far from clear whether appropriate or even effective screening methods were available or even legally permitted (they are currently banned in at least one state in the Australian Federation). The difficult and perhaps unanswerable question for the judges to decide was whether a non-life can be balanced financially with a life of disability. In the UK, a case of a child also born disabled following maternal rubella infection was thrown out of the Court of Appeal because the court found “it was impossible to assess damages because it involved comparing with non-existence, a life with disabilities” (Spencer, 2002). On the other side of the coin, a deaf

lesbian couple wishing to have a child recently arranged privately for a deaf sperm donor (having been refused by clinics) on the grounds that they would find it easier to raise a deaf child than a hearing one. In this case the ethical dilemma of deliberately creating a “handicapped” child caused a media feeding frenzy—although of course many deaf people deny that they have a disability worth discriminating against (BBC news, April 8, 2002: (http://news.bbc.co.uk/hi/english/health/newsid_1916000/1916462.stm).

In Australian case, the presiding judge dismissed the case, however in the perspective of the present review the question is tricky, and could lead to interesting precedents. Gender, height and skin colour can all have an impact on life prospects. Do children have the right to sue for damages if they are born the “wrong” colour? Female? Too short? Not as intelligent as others? Not born to rich parents? Theoretically, all of these factors could be affected by medical decisions made before birth. Even more tricky ethical questions lurk around the corner. If we are able to set a value on an embryo’s chances of establishing a pregnancy, is a clinic legally culpable if it deliberately replaces “substandard” embryos, knowing that these are not likely to succeed? This scenario actually affects all clinics today, as around 70-80% of all assisted reproductive technology (ART) cycles in the USA and Australia are failures (Hurst and Lancaster, 1999, 2001). On a side note, this supposed “failure” of ART is frequently trumpeted by opponents of the technology, who conveniently fail to balance this point with the reality that at least half of all “natural” conceptuses also die, almost certainly because of genetic abnormalities (Short, 1979; Edmonds et al, 1982; Smart et al, 1982). Indeed, some argue that much currently fashionable (and expensive) “treatment” for recurrent miscarriage may be misguided, as pregnancy loss is frequently part of a natural “quality control” measure to eliminate abnormal embryos (Hill and Scott, 2002; Quenby et al, 2002). If we are indeed able to “rescue” dysfunctional oocytes and embryos in an IVF setting (the topic of this chapter), then is a clinic negligent if it fails to do so?

For embryos in the ART laboratory, “rescue” can be seen from two perspectives: rescue from inherent defects such as putative genetic or cytoplasmic disorders, and—probably much more likely to be realized—rescue from deficient culture conditions. The topic is particularly relevant. Recent reports (Brenner et al, 2000, 2001; Barritt et al, 2001a,b.) claim remarkable success in achieving pregnancies for infertile women with a history of poor oocyte and embryo quality, by microinjecting cytoplasm from donor oocytes along with the husband’s sperm. A twin term pregnancy has also been reported after the transfer of cytoplasm from frozen-thawed donor oocytes (Lazendorf et al, 1999). An alternative approach has also been suggested for the transfer of germinal vesicles from older women into “young” enucleated oocytes with the

specific aim of reducing age-related aneuploidy (Takeuchi et al, 2001). In the USA, these procedures have been put on hold as putative gene therapy by the Food and Drug Agency, and will be discussed further below.

WHAT IS A “NORMAL” EMBRYO?

It is relatively easy to define an abnormal embryo as one that does not develop or as one that develops into an abnormal individual (note my previous caution on this point, however). For newborn infants we have good information as there is a well-known and defined risk of birth with a developmental anomaly such as spina bifida, or with a genetic disease (Lechat and Dolk, 1993). With around one million ART children now born world-wide (Schultz and Williams, 2002), the emerging data show that the procedures appear to be associated with an increased general risk of low and very low birth weights, sex chromosome anomalies, multiple major defects and chromosomal and muscle-skeletal defects, particularly after intracytoplasmic sperm injection (ICSI; Hansen et al 2002). One study found limited evidence of mildly retarded development in ICSI children but was not particularly well controlled (Bowen, 1998). Another found a threefold increase in the risk for cerebral palsy, unrelated to birth weight or multiple pregnancy status (Stromber et al, 2002). However, one long-term follow up on a much larger series found no significant difference between ICSI and IVF infants (Bonduelle et al, 2002).

While this appears to point up the long-term risks of ART throughout gestation, possibly reflecting the characteristics of infertile couples as well as the technique per se, what do we know about the embryos themselves? Unfortunately, the definition of a “normal” embryo is elusive. Larsen’s “Essentials of Human Embryology” does not even use the word, although it gives plenty of examples of clinical anomalies (Larsen, 1998). Before we can even attempt to define normality it is therefore necessary to take a step back and look at the molecular biology of development, as without a basic understanding of these processes, any attempts to define normality and abnormality are futile. In addition, attempts to “rescue” embryos without actually defining a specific deficiency is fundamentally non-scientific.

All life forms on earth clearly share a common heritage of transmission of genetic information via the nucleic acids. There are also common patterns and genes involved in controlling development through nested hierarchies of regulatory mechanisms, even in organisms separated by hundreds of millions of years of evolution (Edwards, 2001). Thus, the gene Pax6 encodes for a protein involved in eye development in groups as disparate as vertebrates, urochordates, annelids, molluscs, nemerteans and flatworms: in other words we share a common developmental “genetic toolkit” (Carroll et al, 2001). The unfolding events of the single zygotic

cell transforming itself into a complex organism with specialized cells, tissues and organ systems are so mysterious that one has to wonder how the “normal” process can succeed at all (Larsen, 1998). With only around 30,000 functional genes in the human, clearly, the unfolding events of development must rely on higher order interactions between genes and families of genes rather than simple one-to-one translation from code to protein (Carroll et al, 2001). While this is not the place for an exhaustive review of mammalian embryogenesis, certain principles need to be stated if we are to consider what, if anything that goes wrong during oogenesis or early embryogenesis can indeed be “rescued.” I will concentrate on mammals, even though most is known for insects and other invertebrates. I will also restrict my remarks to the mature oocyte, even though the story of how oocyte “quality” is established starts much earlier, perhaps even when primordial germ cells first invade the presumptive gonad (Edwards, 2001; McLaren, 2001).

The oocyte is released as the equivalent of a piece of biological clockwork, wound up and primed to spring into action once activated by the fertilizing spermatozoon (or by equivalent artificial activating agents) (Saunders et al, 2002). Thus, the dominant founding genotype is that of the mother, and zygotic genes do not start to be actively transcribed in mammals until cleavage is well under way (Larsen, 1998). At a genetic level, regulation and activation of the genes controlling tissue-specific differentiation requires at least three components: RNA polymerase II and associated general transcription factors, cell-specific, tissue-specific, field-specific and signal transducer-type activators; and finally co-activators that regulate that above and influence the local state of the DNA and chromatin (Carroll et al, 2001).

The mammalian oocyte, like those of other better-studied vertebrates such as amphibians, clearly has a pattern of organization that precedes sperm entry and the first zygote division. Indeed the embryonic planes and axes may well be established by gradients of transcription factors and molecules such as leptin (Antczak and Van Blerkom, 1997; Edwards, 2000, 2001a,b; Gardner, 2001, 2002) that in turn are established by spatial positioning and physiological gradients within the developing follicle (Van Blerkom, 1998). There have been claims that the point of sperm entry marks the first cleavage plane (Piotrowska and Zernicka-Goetz, 2001; Plusa et al, 2002). However, the methodology used (attachment of lectin-coated microbeads) has been criticized (Edwards, 2001a) as being non-specific and taking no account of the known tendency for movement of the cell cortex towards the cleavage furrow before cytokinesis in cells (Wang et al, 1994) and in sea urchin zygotes (McCaig and Robinson, 1982). Moreover, it is known that the fertilization cone that marks the sperm entry point is deficient in lectin receptors (Maro et al, 1984). What is truly remarkable is that while sperm entry appears to be random: the

oocyte can compensate for this by rotating its cytoplasm to align its axis with the sperm-derived centrosome (Edwards and Beard, 1997; Payne et al, 1997; Edwards, 2001a). This now establishes the meridional plane of the first cleavage, aligned with the animal-vegetal axis. One the two cells formed cleaves meridionally while the other divides equatorially, resulting in a tetrahedron arrangement of cells. This results in an unequal apportionment of cytoplasmic gradients and possibly, determination of which blastomere will be more likely to contribute eventually to the inner cell mass. There is still no agreement as to whether the orthogonal nature of the second cleavage plane results from the rotation of one blastomere (Gulyas, 1975) or to internal reorientation of one of the spindles. While there are disagreements about the details, it is clear that the left-right, dorso-ventral and antero-posterior axes of the embryo are well defined by the blastocyst stage and that the events that determine this patterning are well in place before first cleavage. What is perhaps surprising is that this appears to be almost universal for the so-called “lower” organisms, so why for many years it was denied for mammals is a scientific puzzle (Gardner, 2001; Zernicka-Goetz, 2002).

Besides internal morphogenetic factors, the oocyte needs to be bioenergetically primed to permit the first set of divisions before the new genome is activated and endogenous metabolism and protein synthesis can start. Of particular importance seems to be the need for appropriately placed and functioning mitochondria with a high inner mitochondrial membrane potential ($\Delta\Psi_m$) actively generating ATP (Cummins, 2001; Van Blerkom et al, 2000,2002). Finally, one important principle is that we are still immensely ignorant about the genetic and epigenetic controls of development, so much so that despite the limited success in cloning animals by nuclear transfer, the majority of animals produced are developmentally abnormal in ways that are mysterious and worrying (Jaenisch and Wilmut, 2001).

WHAT PRACTICAL STEPS ARE AVAILABLE?

GENETIC SELECTION?

As mentioned earlier, ART appears to be associated with an increased risk of birth defects (Hensen et al, 2002; Stromberg et al, 2002). ICSI in particular is linked with sex-chromosome aneuploidy, possibly paternally-related in cases where men have severe infertility (Koulischer et al, 1997). Cox et al. (2002) reported two children with Angelman syndrome, possibly due to a maternal genomic imprinting defect around the time of fertilization. They pointed out that ICSI bypasses many regulatory and selection steps in normal fertilization; moreover, there is evidence from animal models that the dynamics of sperm decondensation in the ooplasm

are altered following ICSI (Terada et al, 2000; Ramalho-Santos, 2000). Similar epigenetic changes leading to fetal overgrowth in lambs are attributed to suboptimal culture conditions for embryos (Young et al, 2001). These problems may go back to the cleavage stage embryo and may be an inherent risk of in vitro culture. Many, if not most (for some women) early IVF embryos are chromosomally mosaic, with a suspected link with mitotic nondisjunction and maternal age (Munne et al, 2002). This is much higher than the 1–2% found in spontaneous abortions or in pregnancies subjected to prenatal diagnosis by chorionic villus sampling (Kalousek et al, 1991). Moreover, there are clear links between differing laboratory conditions and rates of mosaicism suggesting that at least part of the causes may be iatrogenic and lie in the in vitro culture itself (Munne et al, 1997). While ethically contentious, it is possible that preimplantation genetic diagnosis using techniques such as whole genome amplification and comparative genomic hybridization followed by embryo selection may allow clinics to weed out abnormal embryos and thus increase the likelihood of normal implantation (Wells and Delhanty, 2000, 2001; Munné and Wells, 2002). Of course, this is not really “rescue” in the context of this chapter—at least in so far as it does not involve germline gene therapy (Resnik and Langer, 2001)—but it does offer some practical solutions, although application of this high level of genetic analysis in routine ART laboratories would seem to be unrealistic at present.

To a certain extent, genetic selection is already happening. Most likely only the “fittest” embryos are likely to survive to term and the tendency to pick the most rapidly growing “healthy” embryos may be more likely to select for males, which according to some reports, grow faster than females and which in some species are less susceptible to suboptimal culture conditions (Kochhar et al, 2001; Gutierrez-Adan et al, 2001; Cross, 2001). If this is also the case for the human species, could it not be argued that for selective purposes, demonstrations of competence in early embryos may be more effective if culture media or conditions were designed to be stringent rather than ‘all-encompassing’: i.e., formulated to promote the early development of all embryos regardless of inherent viability? The need to balance success in terms of achieving a pregnancy against the risks of multiple pregnancies has led to intense pressure to develop better means of predicting oocyte and embryo “quality”. However, as this is not strictly speaking embryo “rescue” but part of good laboratory practice (Gianaroli et al, 2000) will not discuss it further.

ZONA DRILLING AND ASSISTED HATCHING?

The zona pellucida is a glycoprotein shell that surrounds and protects the oocyte and acts as a species-specific target for the penetrating spermatozoon. After fertilization, it normally thins and weakens through

lysins from the developing embryo (possibly with some assistance from the uterine environment) as well as through physical expansion, and it finally allows the embryo to escape and implant in the endometrium. Zona hardening by proteolytic action from cortical granules is one natural sequel to fertilization that acts to minimise polyspermic fertilization (Yanagimachi, 1994), however it may also be induced by suboptimal conditions and aging in vitro. First suggested as a means of alleviating "excessive hardness" (Cohen et al, 1988), a number of approaches have been suggested ranging from thinning with acidified Tyrodes solution, partial dissection and even laser-assisted drilling (Mandelbaum, 1996); see Wright and Jones, this volume). It should be noted, however, that zona 'hardness' is largely a subjective designation rather than an objective one that can be defined by analytical methods that can accurately measure this parameter. Indeed, a recent meta-analysis by De Vos and Van Steirteghem (2000) pointed out that objective evidence for "excessive hardness" of the zona is hard to obtain. The inclusion criteria for patients believed to possibly benefit from this procedure include increased female age, "excessive" zona hardness or thickness, "poor quality" oocytes and embryos, and previous repeated IVF failure. Another possibility linked to poor embryo quality might be the under-production of embryonic lysins to allow for hatching. While mouse models appear to be safe and to give rise to normal young, there is little evidence that assisted hatching has any benefits at all, except for a limited subgroup of women aged over 38 with a combination of poor prognosis embryos and elevated basal FSH levels (Cohen et al, 1994). There is weaker evidence of a benefit for women with repeated IVF failure (Antinori et al, 1996). Thus I have to conclude that zona manipulation as a means of embryo "rescue" is far from proven: it may simply reflect that clinics still rely on sub-optimal culture conditions—a topic I refer to below.

OOPLASMIC ENHANCEMENT?

Two approaches appear to have been used: ooplasmic transfer with the aim of "improving" the cytoplasmic quality, and the removal of potentially toxic cytoplasmic fragments and debris. For the first, recent reports (Brenner et al, 2000, 2001; Barritt et al, 2001a,b) claim remarkable success in achieving pregnancies for infertile women with a history of poor oocyte and embryo quality by microinjecting cytoplasm from donor oocytes along with the husband's sperm. A twin term pregnancy has also been reported after the transfer of cytoplasm from frozen-thawed donor oocytes (Lanzendorf et al, 1999). An alternative approach has also been suggested for the transfer of germinal vesicles from older women into "young" enucleated oocytes with the specific aim of reducing age-related aneuploidy (Takeuchi et al, 2001).

As reported in the World IVF Congress in Buenos Aries (17 March 2002: http://www.ferti.net/fertimagazine/congress/2002_WIVF_01.asp#01)

"Up until June 2001, 28 patients who were egg donation candidates had undergone 33 cycles of ooplasmic transfer by injection. An egg donation cycle with synchronous follicular stimulation for patient and donor was used, and ooplasm could be extracted from a single donor oocyte up to a maximum of three times without compromising the outcome. The patients had recurrent implantation failure, with poor embryo morphology. The 33 attempts gave rise to 13 clinical pregnancies and a total of 17 babies (including one set of twins and one quadruplet). Comparing implantation rates from previous cycles with those achieved after ooplasmic transfer does indicate possible hope for the technique but it remains difficult to make a statistically relevant assessment."

Work ceased in July 2001 when the FDA decided that ooplasmic transfer is a form of gene therapy and that approval would be needed. An FDA commission dealing with gene therapy met in May 2002 to decide whether or not these experimental protocols are acceptable under certain conditions. (see <http://www.fda.gov/>). This approach drew strong criticism from the outset; mainly focusing on the mitochondrial issue, as an abstract of early report rather brashly announced, "This report is the first case of human germline genetic modification resulting in normal healthy children" (Barritt et al, 2001a). Clearly there is no evidence yet for any germline transmission as the children are far too young to reproduce! While ooplasmic transfer in mice can overcome a strain-specific two-cell block in development (Muggleton-Harris et al, 1982; Pratt and Muggleton-Harris, 1988), the only animal study that appears directly relevant showed that microinjection of normal cytoplasm into peroxide-stressed eggs, with compromised mitochondrial function, partially restored their ability to develop (Liu and Keefe, 2000). The same two-cell block in the mouse can also be overcome by using different culture media (see Biggers, this volume), which demonstrates that this phenomenon is an artifact of in vitro culture and not an inherent developmental defect. The movement of this invasive approach into ART clinical practice, particularly as no mitochondrial deficiency was demonstrated in the recipients (see Brenner, this volume), has been criticized as lacking scientific rigor and suitable controls or relevant animal models (Robertson, 1999; Thorburn et al, 2001; Hawes et al, 2002; Templeton, 2002). While vigorously defended by its main proponents (Malter and Cohen, 2002) some eminent reproductive biologists, such as Roger Short, Alan Trounson and Lord Winston have publicly expressed their skepticism as to the real benefits, if any, of cytoplasmic transfusion. One pungent criticism was that the procedure "may be akin to trying to improve a bottle of spoiled milk by adding a cup of fresh" (Hawes et al, 2002). Moreover, at least for mitochondrial (mt) DNA there is

considerable doubt that the technique originally reported (fluorescence DNA sequencing) is sensitive enough to detect levels of heteroplasmy less than 30%, and yet the original oocyte received at most, only about 15% of donor cytoplasm (Thornburn et al, 2001). These investigators point out that that a technique such as "last hot cycle" PCR-RFLP analysis (White et al, 2001) would be more appropriate for the quantitation of small amounts of donor mtDNA.

While clinical trials on such limited numbers are notoriously hard to control scientifically, in these particular reports there are so few appropriate controls that it is hard to know what was going on. For example, would the simple act of aspirating cytoplasm and reinserting it with the sperm have the same 'tonic' or 'restorative' affect of improving embryo development? What of inserting cytoplasm from mitochondrial-free cytoplasts (Van Blerkom et al, 1998)? Apart from mitochondria, the transferred ooplasm is sure to contain a rich cocktail of RNAs, enzymes, growth factors and other bioactive molecules that could affect development. The proponents themselves do not claim to know the secret, so we are left dangling in a scientifically and medically unsatisfying state. Small ART studies are notorious for the statistical flaw of regression to the mean, which occurs whenever samples or subjects are chosen on the basis of extreme pre-test scores (in this case, poor embryo development). Results in these cases are statistically expected to improve, particularly when there is non-random selection of subjects (Shaugnessy and Zechmeister 2001; Silber, 2001).

One basic objection to cytoplasmic transfer, at least from the point of mtDNA, is that it deliberately creates heteroplasmy (multiple mtDNA genotypes). This state is often associated with mitochondrial diseases (Chinnery et al, 2000). Moreover, mismatch between mitochondrial and nuclear genomes may lead to problems in bioenergetics (Cummins, 2001a,b; 2002) and at least one mouse model shows impaired neuromuscular function (Nagao et al, 1997). On the other hand, the association of disease with heteroplasmy does not imply cause-and-effect: many animal models created by nuclear transfer, cytoplasm or karyoplast transfer have normal development (Smith et al, 2002). Nature has a way of surprising us, however, and chance occurrences should alert us to the potential risks of tampering with cytoplasmic inheritance in early development. A case was recently reported of a young man with severe exercise intolerance due to lactic acidosis and with severe ragged-red muscle fiber characteristic. It was discovered that 90% of his mtDNA was actually paternal in origin with a de novo 2-bp mutation in the ND2 gene (complex I of the respiratory chain), while his blood cell mtDNA was maternal (Schwartz and Vissing, 2002; Williams, 2002). The authors speculated that one or more sperm mitochondria must have eluded the normal seek-and-destroy ubiquitin-proteasome system (Sutovsky et al,

2000) and somehow proliferated preferentially. Clearly the strict dogma of maternal inheritance (Elson et al, 2001) was bypassed in this instance, and I suspect that it may be more common than currently recognized, as the very existence of mechanisms aimed at eliminating paternal mtDNA means that these can occasionally fail, but we do not register the fact, as pathogenic states are eliminated early in embryogenesis (Hagelberg, personal communication).

The other approach that has been advocated for “enhancing” embryo quality is to remove potentially toxic fragments or cell debris, either occurring spontaneously during culture (Alikani et al, 1999) or as a result of laboratory-induced damage following cryopreservation. For the latter, the presence of damage clearly reduces implantation potential (Van den Abbeel et al, 1997), and laser-assisted removal of necrotic blastomeres from frozen-thawed embryos coupled with zona drilling has been claimed to enhance pregnancy rates significantly (Rienzi et al, 2002). While removal of necrotic, damaged or lysed cells caused by cryodamage has logic, the question of spontaneous fragments is less clear-cut. These structures may be normal and transient during embryogenesis and can disappear spontaneously—particularly if they lack mitochondria—and do not necessarily affect potential (Alikani et al, 1999; Antczak and Van Blerkom, 1999; Van Blerkom et al, 2001). Whether these features reflect inherent defects in embryos or defective culture conditions (below) is still not clear.

IMPROVING CULTURE CONDITIONS?

As mentioned above, there is a strong impetus in ART to reduce the incidence of multiple pregnancies with their associated high morbidity while still maintaining an overall high pregnancy rate (see Gerris, this volume). A report in *New Scientist* (16 July 2001) suggests that “If current trends for triplet births continue, almost a third of all people born will be a triplet within a decade or so.” In the USA a staggering 36.6% of all IVF births are multiples (Anonymous, 1999) with an additional cost (in year 2000) of US \$640 million (Collins, 2001). Some argue that we should be moving towards single embryo transfer as a matter of course and one key to this may be improving the culture conditions (Templeton, 2000; Biggers, this volume). Despite these cautions, clinics still feel impelled to claim miraculous results based on a “league table” of crude pregnancy rates regardless of the life-threatening nature and costs of multiple pregnancies (Westphal, 2002). Indeed, when one considers the differences between the environment of the human embryo *in vivo* with what we expose it to *in vitro*, the surprise to some may be that any survive at all. However, human IVF did not develop in a vacuum or from first principles. Rather, culture media and methods used to fertilize and

develop human embryos tapped into a vast reservoir of experience from animals systems that dates back at least 50 years (see Biggers, this volume) and which have produced normal offspring. In common clinical practice, human IVF embryos never experience the environment of the oviduct, and traditionally have been transferred to the uterus during the cleavage stages after two or three days of culture, even though implantation normally occurs at the blastocyst stage after day six. In the past, this may have reflected clinics' acknowledgment that culture conditions were sufficiently inadequate so that replacing embryos as soon as possible was an acceptable trade-off to achieve a commercially viable pregnancy rate, albeit with some proportion resulting in higher order gestations. In reality, the preimplantation embryo develops through at least two major phases: an early undifferentiated one largely controlled by existing pools of maternal RNA, and a later stage when growth and responses to external factors commences along with differentiation and genome activation (Leese, 1995; Pool, 2002).

While the decision on timing of embryo transfer may still be a trade-off, there is now a strong push to transfer embryos at the blastocyst stage; in theory allowing the "best" embryos to be selected and thus reduce the risks of multiple pregnancies (Gardner et al, 2002). This is far from established practice, however, as if embryo quality is already compromised a period of extended culture may make matters worse, and it is not certain which patient groups will benefit the most (see chapters by Gerris and Viega, this volume). One prospective randomised study found that blastocyst transfer on day 5 gave significantly worse pregnancy rates than embryo transfers on day 3 (Levon et al, 2002). Others found the opposite (Milki et al, 2000; Van Der Aywera et al, 2002) or no difference (Coskun et al, 2000). One problem with blastocyst culture is that it appears to significantly increase the rate of monozygotic twinning for reasons unknown (Behr et al, 2000), so even single embryo transfer cannot totally avoid the problems of multiple gestations. An alternative to blastocyst transfer for avoiding multiple pregnancies is rigorous selection and single embryo transfer with cryopreservation of the surplus (see Gerris, this volume). One study found a cumulative live-birth rate of 53% per oocyte retrieval (Tiitinen et al, 2001). Incidentally, selection of "good" blastocysts for transfer does not necessarily eliminate gene defects as paternally inherited defects may not be apparent at this stage (Banerjee et al, 2000).

Initially, IVF was carried out in media developed for somatic cell culture such as formulations based on Earle's Balanced Salt Solution and subsequently worked up in animal models (Bavister, 2001; Biggers, 2001, and this volume). Media developed specifically for human IVF started to be marketed in the 1980s (Menezo et al, 1984; Quinn et al, 1985), but were really only suitable for develop through the early cleavage stages.

Co-culture in the presence of monolayers of animal or human cell lines was widely used in attempt to improve the growth rates to the blastocyst stage (Menezo et al, 1995; Wiemer et al, 1998). It was argued that the move to sequential culture media was based on the experiences emerging from co-culture (Menezo et al, 1998) even though culture with largely undefined primary cell lines of various sources (including non-human) failed to identify any specific factor or factors that might be missing in conventional media. Moreover, the very use of such undefined cell lines was criticized as inappropriate and possibly dangerous given our new knowledge about prions and viral transmission, especially when using slaughterhouse material, including from bovine sources, that was not archived for subsequent reference. Indeed the rationale for using co-culture was that it “improved” the rate of growth to blastocyst even though the evidence that simple cell numbers are “better” is weak. It is worth remembering that statistical significance does not necessarily equate with clinical significance particularly when the underlying biochemistry and physiology are undefined (Van Blerkom, personal communication).

The new formulations in culture media are aimed at mimicking the changed environments embryos experience during their passage from the high pyruvate, low glucose environment of the oviduct to that of the uterus (high glucose, low pyruvate) and including appropriate amino acids and to some extent, growth factors (Menezo et al, 1998; Langley et al, 2001; Gardner et al, 2000, 2002; Pool, 2002). The validity of this approach and the accuracy and utility of measurements of metabolites in the female reproductive tract have been questioned by Biggers (this volume). Other approaches involve reducing oxidative stresses by including EGTA and culturing in a low partial pressure (5%) of oxygen (Pool, 2002; Schultz and Williams, 2002). In addition, what might appear minor alterations to laboratory conditions, such as improved temperature control during ICSI (Wang et al, 2002), culturing in micro-drops (Jones et al, 1998) or micro-channels (Pool, 2002²⁹⁷), or more stable incubator systems (Vajita et al, 1997) can all have significant affects on embryo potential in vitro. Emerging microfluidic systems allowing nearly complete control of embryo culture media and gas phase in very small environments promise even better systems for embryo culture in vitro (Pool, 2002; Glasgow, 2001; Beebe et al, 2002). While all of these may improve embryo performance in vitro, changes in the design and physical properties of embryo transfer catheters combined with ultrasound guidance can also be significant ‘non-biological’ factors that improve outcome in IVF.

RESCUE OF GAMETES?

The burgeoning interest in cloning and stem cell technology—together with ready access to the equipment required—has inevitably has its impact on ART and various possibilities have emerged. These include “semi-cloning” or “correction” of age-related aneuploidy by combining a haploidized somatic cell nucleus with an enucleated donor oocyte (Tsai et al, 2000; Palermo et al, 2002a,b; Tesarik, 2002), the recreation of chimaeric embryos by combining viable blastomeres from otherwise non-viable embryos (Alikani et al, 2002), and the maturation of immature sperm *in vitro* (Tesarik et al, 1999, 2000a,b; Tesarik and Greco, 1999; 126-129) or even in allografts to animal hosts (Brinster, 2002). While reproductive cloning *per se* is generally seen as unethical or inadvisable in today’s political climate, these proposals at least have the potential to shed experimental light on the nature of abnormal embryo development and differentiation. Unfortunately, the discussion of dispassionate research proposals is not always understood by the public or politicians, who often think that because scientists discuss a possibility this means that implementation is automatic.

ARGUMENTS FOR EMBRYO OR GAMETE RESCUE

Arguments for embryo or gamete rescue are usually based on the premise that infertile couples have an absolute entitlement to a genetic child of their own, regardless of the costs or possible problems therein. However, does this mean that every embryo, regardless of quality, potential or genetic make-up, is potentially capable of being rescued? I personally think this contradicts biological reality, and I feel uneasy that the offer of hope to desperate couples may sometimes be inappropriate. The notion of “rescue” tends to be couched in absolutist terms that touch on deep ethical fault lines underlying the debate over abortion and “right to life”, even though there is no universal agreement on the moral or ethical status of the human embryo (Reiss, 2002). While clinics may argue for a couple’s right to have a child at any cost, the interests or potential interests of the child are not often highlighted. In the case of the deaf lesbian couple deliberately selecting a deaf sperm donor, I personally find it hard to accept the argument that this was for the benefit of the child: rather than for the convenience of the parents. In such a case it is difficult to counter the argument that ART is being used to produce a child as a commodity. I find little sound evidence that the approaches to embryo or gamete “rescue” discussed above meet the objective criteria of evidence-based medicine, with double blind randomized trials based on clear and unambiguous selection criteria and science-based objective hypotheses. ART is just too messy, emotive and open to exploitation, as for example

the recent history of testosterone replacement “therapy” being advocated for aging males in the absence of good evidence of hormone deficiency demonstrates (Handelsman, 2002). The sloppy reporting over the supposed decline in human sperm counts (Handelsman, 2000), the weak justification for the use of highly expensive immunotherapy for recurrent miscarriage (Hill and Scott, 2002), and the controversial use of adjunct treatments such as acupuncture or even prayer (Reenckens, 2002; Stener-Victoria et al, 2002) offer other examples.

As I mentioned earlier, the commercial pressures on clinics to achieve ART pregnancies at any cost (Westphal, 2002), coupled with a highly charged atmosphere in which couples may experience extreme psychological distress, can lead to distortions of sound judgement when faced with difficult reproductive choices. Thus, even when faced with the prospect that their sons will bear the same genetic condition (Y chromosome deletions) that cause their own infertility, many men insist on proceeding with ART using testicular sperm (Cram et al, 2000; Tse et al, 2001). I suspect that here we have the analogy of the timid diver on a high platform, too abashed to admit fear. By the time couples reach the stage of accepting the need for such highly invasive ART they have so much emotional energy—and money—invested in the process they are unwilling or unable to go back.

There is a fundamental question that IVF clinics need to address as they contemplate the introduction of new therapies and untested invasive procedures for oocytes and embryos where an underlying defect is suspected but unknown, or where the potential for adverse downstream consequences exists but may not be recognized: who is the patient, the infertile couple or the child they want so much to produce? There is little evidence that ART clinics are prepared to offer objective evaluations of their various claims for miraculous results. A recent survey of Internet sites offering advice on infertility found that only 2% met core standards of responsibility in terms of disclosing authorship or commercial interests, citing the peer-reviewed literature or even ensuring that the information was current (Okamura et al, 2002). This is particularly worrying given that individuals appear much more likely to seek Internet information about infertility than other health matters (Weissman et al, 2000).

Obviously there has to be a way forward. ART clinicians must avoid the temptation to be miracle workers or shamans. If we are to offer a service to assist infertile couples it must be based on the tried tenets of scientific method including the acceptance of biological imperfection lest this important and fundamentally beneficial enterprise become tainted by false promises and descriptors such as “voodoo fertilisation”. Attempting to market the notion that all embryos are potentially equal and thereby somehow capable of being “rescued” has the potential for litigious backlash on the industry, as disgruntled would-be parents take their

revenge. I am reminded of the notion of hubris in classical Greek philosophy: overweening pride and presumptive disregard of the limits of human action. Unfortunately, hubris leads to nemesis.

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CHAPTER TWENTY ONE

DETERMINATION OF ENDOMETRIAL STATUS AND THE IMPLANTATION WINDOW

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INTRODUCTION

Human embryo implantation normally takes place between days 20 and 24, or between days LH +7 and LH +11 of a normal conception cycle. The process begins with apposition of the hatched blastocyst to the luminal surface epithelium of the endometrium and is followed by adhesion, invasion and anchorage of the trophoblast into the endometrial stroma where it comes into contact with maternal blood vessels. Irrespective of whether fertilization takes place *in vivo* or *in vitro*, endometrial receptivity results from an orchestrated interplay between the embryo and the maternal endometrium in which hormones, hormone receptors, oligosaccharides, lectins, integrins, growth factors, cytokines, proteases and their inhibitors, prostaglandins, peptides, and factors that modulate immune cell reactivity against the embryo play a role. At the same time, the endometrium provides constraints on uncontrolled invasion of the trophoblast. A receptive endometrium is an end result of responses to endocrine and paracrine messages that derive from the stimulated ovary, exogenous hormones, endometrium, and the embryo. For instance,

Abbreviations: AR, androgen receptor; COH, controlled ovarian hyperstimulation; E2, estradiol; ER, estrogen receptor; hCG, human chorionic gonadotropin; GnRH, gonadotropin-releasing hormone; HB-EGF, heparin-binding epidermal growth factor; HRT, hormone replacement treatment; IFN, interferon; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; IL-1, interleukin-1; IL-1R_{tL}, IL-1 receptor type I; IL-1ra, IL-1 receptor antagonist; LIF, leukemia inhibitory factor; LIFR_b, LIF receptor β; LH, luteinizing hormone; MMP, matrix metalloproteinase; NK cell, natural killer cell; PR progesterone receptor; PCOS, polycystic ovary syndrome; PEP, progesterone-associated endometrial protein; PP14, placental protein 14; TGFβ, transforming growth factor β; TIMP, tissue inhibitor of metalloproteinase; Th1 and Th2 cells, T helper 1 and -2 cells.

poor oocyte quality may be associated with inadequate follicle maturation and hormone secretion that affect embryo quality, endometrium and implantation.

ENDOMETRIAL FUNCTION AND STROMAL/EPITHELIAL/ EMBRYONIC INTERACTIONS

Ovarian steroids initiate changes in growth factors and cytokines that are produced locally in maternal endometrium. The endocrine stimuli from blood arrive first to the endometrial stroma from which the messages are transmitted to the epithelium and luminal surface. Studies using null mouse mutants for ER and PR have demonstrated the primary role of endometrial stroma in directing proliferation and differentiation of the overlying epithelium (Cooke et al., 1997; Kurita et al., 1998). Regulatory substances from the extracellular matrix play an important role in this communication (Arnold et al., 2001; 2002), and human endometrial stroma has been shown to express growth factors such as HB-EGF that induces differentiated functions in the endometrial epithelium (Lessey et al., 2002a).

During implantation, there is a dialogue between the embryo and the endometrium in which the embryo receives and sends signals to the endometrium (Liu et al., 1995a; Simon et al., 1997a; Simon et al., 1997b). In the apposition phase, the presence of a blastocyst rescues the epithelial cells from apoptosis, but when the human blastocyst adheres to the epithelial cells, it induces paracrine apoptotic reactions in these cells (Galan et al., 2000). Immunostaining has confirmed the presence of IL-1, IL-1R α , and IL-1ra in human oocytes and embryos. The differential role of endometrial epithelium and stroma has been demonstrated in cocultures showing that, when embryos are cocultured with stromal cells, IL-1 α , IL-1 β , and IL-1ra are absent in conditioned media, whereas when cocultured with endometrial epithelium, two different embryo populations are identified: IL-1 producers (57%) and IL-1 non-producers (43%). Endometrial epithelium appears to have an obligate role in embryonic IL-1 production, as endometrium-stimulated IL-1 production is important for the embryo to implant (De los Santos et al., 1996). The IL-1 intracavitary microenvironment surrounding the human embryo is particularly important for the apposition phase (Simon et al., 1997a and b).

In extracellular matrices and blood, many adhesive proteins contain the tripeptide arginine-glycine-aspartic acid (RGD) sequence that serves as their cell recognition site (Ruoslahti and Pierschbacher 1987). This sequence has been suggested to be critical for the attachment and outgrowth of the trophoblast (Armant et al., 1986).

MARKERS OF UTERINE RECEPTIVITY

ULTRASOUND

Ultrasound examination is routine in today's management of infertility. In the proliferative phase, there is a typical triple-line pattern which disappears in the secretory phase and the endometrium becomes hyperechoic. Estrogen increases endometrial thickness, whereas treatment with antioestrogen results in a thinner endometrium. Stimulation with human menopausal gonadotrophin either alone or in combination with a GnRH agonist makes little difference and the resulting thickness is not predictive of pregnancy. Implantation may require a minimum endometrial thickness, as higher pregnancy rates have been observed in the women whose endometrial thickness is greater than 7.5 mm compared to those with a thinner (< 5 mm) endometrium (Abdalla et al., 1994). However, beyond this threshold there is no clear relationship between endometrial thickness and implantation rate. Normally, resistance to blood flow decreases in the mid-luteal phase, indicating that uterine artery blood flow is increased when implantation takes place. Many studies have shown that infertile women have higher uterine artery impedance, and an inverse correlation has been observed between the uterine artery pulsatility index and the conception rate. The mean pulsatility index and the resistance index are lower in conception cycles compared to nonconception cycles, but when used alone they have low positive predictive value (Steer et al., 1992; 1995).

MORPHOLOGICAL CHANGES

Clinical assessment of endometrial responses to hormonal stimuli has long rested on endometrial histology of a biopsy specimen. But, where pregnancy is desired, invasive methods may jeopardize the implanting embryo. For historical reasons endometrial biopsy is taken in the late luteal phase. Biopsies performed during the temporal window of implantation may detect a greater number of women with delayed maturation (Castelbaum et al., 1994). There is a positive correlation between morphological maturation and functional capacity of the endometrium, but adequate morphology does not necessarily mean normal receptivity (Younis et al., 1996). Glandular-stromal dys-synchrony has been described by most investigators reporting on cycle day 21-23 histology in estrogen and progesterone supplemented cycles of donor-egg IVF recipients. Accelerated maturation is more common in the stroma than in the glands, and accelerated vascular maturation is also common. Well developed spiral arteries are important for uterine receptivity.

ULTRASTRUCTURAL CHANGES – THE PINOPODES

At the time of implantation, the apical membranes lining the uterine cavity loose their microvilli and develop large and smooth membrane projections named pinopodes because of their pinocytotic activity. Fully developed pinopodes may facilitate contact between uterine epithelium and embryonic trophectoderm. Their development is progesterone-dependent and they have a short life span of less than 48 h coinciding with the implantation window as shown by sequential endometrial biopsies (Nikas and Psychoyos, 1997). However, two recent prospective studies suggest that pinopodes appear during the window of implantation and remain throughout the luteal phase (Acosta et al., 2000; Usadi et al., 2003). Up to 80% of endometrial biopsies obtained from normally ovulating women show pinopodes on the sixth post-ovulatory day. The most frequent appearance of pinopodes is on day 19 in COH cycles, on day 20 in natural cycles, and on day 21 in HRT cycles (Develioglu et al., 1999). The most predictive feature of premature expression of pinopodes is the onset of progesterone secretion.

STEROID RECEPTORS

In a normal menstrual cycle, estrogen stimulates PR and progesterone downregulates PR in the glandular compartment (Lessey et al., 1988). The establishment of normal endometrial receptivity appears to be tightly associated with the downregulation of both ERA (Lessey et al., 2002b) and epithelial PR (Lessey, 1997). During the secretory phase, expression of ER α declines in the glands and the stroma of the functionalis. ER β also declines in glandular cell nuclei but not in the stroma (Critchley et al., 2001). Levels of expression of ER α and ER β in all cellular compartments remain unchanged in the basalis throughout the menstrual cycle. However, there is a striking contrast between the pattern of expression of ER α and ER β in the vascular endothelium; only ER β is present in the endothelial cell population. How the fine-tuning of the subtype expression relates to endometrial receptivity remains to be assessed.

Estrogen receptor over-expression may be a common finding in women with suspected defects in endometrial receptivity. These would include women with endometriosis (Lessey et al., 1994b), PCOS (Lessey et al., 2000; Apparao et al., 2002), and luteal phase defects (Lessey et al., 2000; 2002b). Estrogen appears to be a potent inhibitor of gene expression during the window of implantation. Examples include $\alpha v \beta 3$ integrin (Somkuti et al., 1997) and decay accelerating factor (DAF; Young et al., 2002). Over-expression of ER α may be due to elevated estrogen levels as

in PCOS, or due to local conversion of estrogen by aromatase, e.g., in the endometrium of women with endometriosis (Bulun and Zeitoun, 2000; Kitawaki et al., 1999). In luteal phase defects, a delay in histologic progression is likely associated with a delay in ER α downregulation.

THE IGF SYSTEM

This system consists of insulin-like growth factors I and II, their receptors, IGF-binding proteins, and proteases that cleave some of the binding proteins, thereby affecting their binding affinity (Jones and Clemmons, 1995). In the endometrium, IGF-I mediates the mitogenic actions of E2 and is abundant from mid-proliferative to early secretory phases, whereas the expression of IGF-II increases in secretory phase endometrium. IGF-II is the major growth factor of the invading trophoblast. It is exclusively expressed in the extravillous trophoblasts that secrete metalloproteinases as they degrade the decidual extracellular matrix during placentation. Importantly, IGF-II inhibits stromal cell tissue inhibitor of metalloproteinase-3 (TIMP-3) and IGFBP-1 (see below) in endometrial stromal cells, thereby inhibiting maternal restraints on trophoblast invasion (Irwin et al., 2001).

The six IGF-binding proteins are characterized with a high binding affinity N-terminal domain (Hwa et al., 1999). IGFBP-1 is one of the major protein products of endometrial stroma during the mid secretory phase, with increasing abundance in decidua (Rutanen et al. 1985; Koistinen et al., 1986; Julkunen et al., 1990). The biological action of IGFBP-1 depends on its phosphorylation status so that phosphorylated IGFBP-1 inhibits IGF actions, whereas non-phosphorylated IGFBP-1 is stimulatory (Jones and Clemmons, 1995). In decidua, the degree of phosphorylation increases as pregnancy advances, resulting in secretion of IGFBP-1 with higher IGF-binding affinity (Koistinen et al., 1993) and inhibitory effects on the binding of the IGFs to their cellular receptors in the endometrium (Rutanen et al., 1988). IGFBP-1 but not IGFBP-3 binds to placental cyto trophoblast $\alpha 5\beta 1$ integrin and inhibits trophoblast invasion into decidua (Irwin and Giudice 1998).

Coculture with human endometrial stromal cells significantly increases the expression of IGF-I, IGF-II, type IGF-I receptor, and the insulin receptor in cocultured human embryos (Liu et al., 1999). This may have significance for implantation through improved embryo quality, as autologous coculture has led to significant improvement in the mean number of blastomeres per embryo and to a decrease in the fragmentation rate compared with noncultured embryos from the same patient (Barmat et al., 1998). As endometrial stromal cells synthesize a number of IGFBPs (Julkunen et al., 1990; Giudice et al., 1991), it is of interest to note that the

IGFBPs in stromal cell cocultures, notably IGFBP-3, are responsive to embryonic signals, whereas IGFBP-1 is particularly responsive to progesterone and relaxin (Rutanen et al., 1986; Liu et al., 1995b). Interestingly, IGFBPs are also present in embryonic cells, suggesting that they have autocrine/paracrine effects on the embryo and the endometrium during the implantation process.

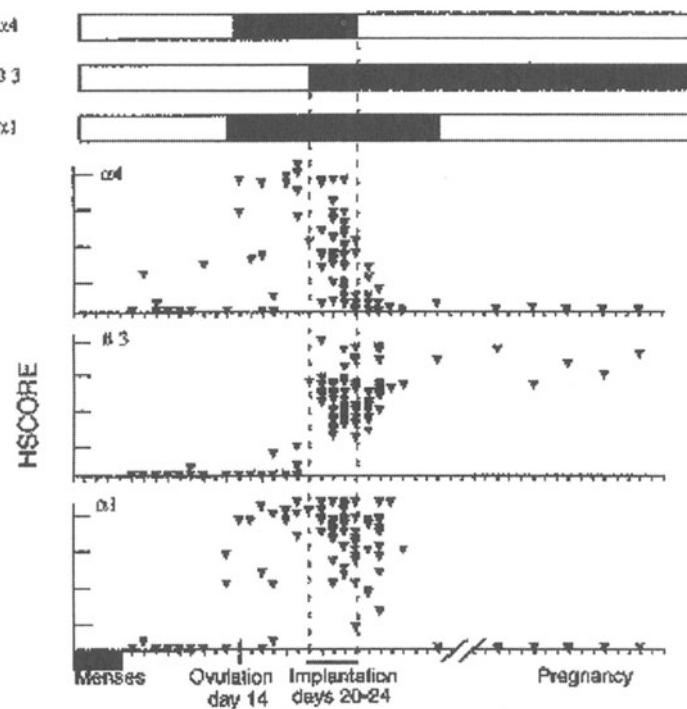


FIGURE 21.1 Relative intensity of staining for the epithelial $\alpha 4$, $\beta 3$ and $\alpha 1$ integrin subunits throughout the menstrual cycle and in early pregnancy. Immunohistochemical staining was assessed by a blinded observer using the semi-quantitative HSCORE (ranging from 0 to 4) and correlated to the estimate of histological dating based on pathologic criteria or by last menstrual period in patients undergoing therapeutic pregnancy termination. The negative staining (open bars) was shown for immunostaining of an average HSCORE <0.7, for each of the 3 integrin subunits. Positive staining for all three integrin subunits was seen only during a 4 day interval corresponding to cycle days 20-24, based on histologic dating criteria of Noyes et al. (1950). This interval of integrin coexpression corresponds to the putative window of implantation. Of the three, only $\alpha v\beta 3$ integrin was seen in the epithelium of pregnant endometrium (Lessey et al., 1994a, used with permission of the American Society for Reproductive Medicine).

THE INTERLEUKIN-1 (IL-1) SYSTEM

The IL-1 system consists of two agonist cytokines (IL-1 α and IL-1 β), two receptors, IL-1 receptor type I (IL-1R tI) and II (IL-1R tII) and the homologous IL-1 receptor antagonist (IL-1ra). The IL-1 receptor increases in early and mid-late secretory phases and appears to interact with embryo secreted IL-1 β during implantation (Simon et al., 1993; Polan et al., 1995). IL-1 β has been localized by immuno-histochemical methods in endothelial cells and isolated stromal cells in the human endometrium throughout the menstrual cycle. In cultured stromal cells from secretory endometrium, IL-1 β increases its own receptor, whereas the constitutive presence of IL-1R tI in endometrial epithelial cells is not affected by IL-1 β . The IL-1 receptor antagonist appears to prevent implantation by downregulating the $\alpha 4$, αv and $\beta 3$ integrins on the endometrial epithelium (Simon et al., 1998a). According to current knowledge, embryo secreted IL-1 interacts with IL-1R type I on endometrial cells. As these events in mice are blocked by the administration of IL-1ra, taken together, these results indicate that the IL-1 system is a requisite for successful implantation (Polan et al., 1995).

ENDOMETRIAL INTEGRINS

Integrins have previously been described in the human endometrium (Lessey et al., 1992; Tabibzadeh et al., 1992) and exhibit both constitutive and cycle-dependent patterns of expression. These heterodimeric glycoproteins consist of α and β subunits that pair in predictable patterns. A pattern of three integrins has been described in which their coexpression precisely matches the suspected time of endometrial receptivity (Lessey et al., 1994a) (Fig. 21.1). These include $\alpha 1\beta 1$ collagen receptor, the $\alpha 4\beta 1$ fibronectin receptor, and the $\alpha v\beta 3$ vitronectin receptor. The latter appears at the time the embryos first are thought to attach, and is expressed on the apical pole of the luminal surface. This integrin recognizes the three amino acid sequence RGD and binds osteopontin, which is coexpressed during the time of maximal endometrial receptivity (Apparao et al., 2001). The loss of integrin has been suggested to correspond to poor receptivity and has been described in various clinical conditions (Lessey et al., 1995; Castelbaum and Lessey, 2001).

EFFECTS OF HCG ON THE ENDOMETRIUM

During clinical IVF, endometrium is exposed to hCG on two occasions, first after the preovulatory administration of hCG and, second, immediately after implantation. Importantly, the embryo starts secreting hCG when cultured *in vitro* for more than 7 days (Fishel et al., 1984). In

addition to its known actions on the corpus luteum, hCG may have effects on paracrine parameters of endometrial differentiation and implantation. In baboons, infusion of hCG into the uterine cavity during the window of receptivity induces the expression of a smooth muscle actin in the subepithelial stromal fibroblasts and increases both the transcription and post-translational modification of glycodelin in the glandular epithelium (Fazleabas et al., 1999). Local infusion of hCG into the uterine cavity of women inhibits IGFBP-1, prolactin and macrophage colony-stimulating factor (M-CSF) expression, and also affects the expression of LIF and vascular endothelial growth factor, VEGF (Licht et al., 1998; 2001). These observations suggest a role for hCG in the control of endometrial receptivity in preparation for implantation. In contrast to modulating the uterine environment *in vivo*, the role of hCG during *in vitro* decidualization has been somewhat controversial. Studies from several groups have demonstrated that either intact hCG or the alpha-subunit induces prolactin production in stromal fibroblasts in synergism with steroids (Tang and Gurpide 1993; Moy et al., 1996; Nemansky et al., 1998). However, these findings have been contested (Kasahara et al., 2001). One possibility for these discrepancies may be related to the types of signaling pathways that were evaluated. Non-gonadal LH/hCG receptors use either cAMP/PKA, PKC or MAPK signaling pathways individually or combine their actions through crosstalk (Rao, 2001). Alternatively, the culture conditions may have affected the expression pattern of the LH/hCG receptor on stromal fibroblast, making them unresponsive to exogenous hormone treatments. Receptor levels in non-gonadal tissues are generally lower than those in gonadal tissues. Multiple techniques for both protein and mRNA analyses have been used so that methodological artifacts have been eliminated. Furthermore, the fact that non-gonadal actions of hCG are receptor-mediated have been confirmed by the use of anti-sense oligonucleotide constructs. Inhibiting the synthesis of the LH/hCG receptor resulted in the loss of the ability of hCG to stimulate COX-2 expression in uterine epithelial cells (Zhou et al., 1999). However, much work still remains to be done to understand the functional significance of extra-gonadal hCG receptor expression and the role of hCG in uterine physiology.

MATRIX METALLOPROTEINASES (MMPS)

These are enzymes that break down endometrial extracellular matrix and are produced and utilized by the trophoblast during implantation. There are three major classes of MMPs classified according to their substrate specificity: 1) collagenases, 2) gelatinases (e.g. MMP-2 and MMP-9) and 3) stromelysins. In the trophoblast, MMP-9 mediates trophoblast invasion, digesting collagen type IV, the major constituent of

the basement membrane (Osteen et al., 1994; Bishof 2001). Stromelysins are involved in growth-related remodeling of proliferative endometrium and their expression is suppressed by progesterone *in vitro*. Decidua contains tissue inhibitors of metalloproteinases (TIMPs) that regulate stromal receptivity. Examples of these are TIMP-1 and -2 that are not cycle-dependent. In stromal cells TIMP-3 mRNA is upregulated by progesterone and TIMP-3 may control trophoblast invasion.

The embryo may communicate with maternal decidua through the GnRH system. GnRH may promote both embryonic development and endometrial receptivity. GnRH has previously been identified and characterized in the cyto- and syncytiotrophoblast and it stimulates hCG secretion. A GnRH agonist can ablate TIMP-1 and TIMP-3 expression without affecting MMP-9, the major enzyme involved in basement membrane degradation. Trophoblastic GnRH may therefore facilitate trophoblast invasion by inhibiting the endometrial TIMP-related inhibitory mechanisms (Raga et al., 1999). This observation may have clinical significance, as the administration of a GnRH agonist during the early phases of embryo implantation in women undergoing IVF is associated with higher implantation rates.

IMMUNE RESPONSES AT THE FETOMATERNAL INTERFACE

IMMUNE CELLS

Successful pregnancy depends upon tolerance of a genetically incompatible fetus by the maternal immune system. Human endometrium contains a full range of immune cells, including macrophages, leukocytes and lymphocytes. The latter include T cells and NK cells that synthesize interferons. Rejection of foreign tissue is a function of T helper 1 cells characterized by secretion of IL-2 and IFN γ . At the fetomaternal interface, the immune reactions are shifted toward a less aggressive Th2-type response, characterized by production of LIF, IL-4 and IL-10. The shift begins in endometrium during secretory phase and is accentuated during pregnancy (Raghupathy 1997; Piccinni et al., 1998). Hormones influence the development of CD4+ helper T cells into Th1 and Th2 effectors during pregnancy. Progesterone promotes the production of IL-4 and IL-5, whereas relaxin promotes the production of IFN γ (Piccinni et al., 2001).

Endometrial NK cells increase from mid-luteal phase to early pregnancy so that 80 percent of the maternal lymphocytes present in human decidua during the early weeks of pregnancy are NK-like cells (King et al., 1996). They are large granular lymphocytes that have a distinctive phenotype (CD3-, CD56bright, CD61-) and lack type III Fc receptors (King et al., 1996; Clark et al., 1994). Women experiencing implantation failure have elevated percentage of circulating CD56+ NK

cells (>12%) than fertile women (3-12%). In women with elevated percentage of CD56+ NK cells and repeatedly failed IVF, administration of intravenous immunoglobulin has improved the pregnancy rate (56%) compared to those women who did not receive immunoglobulin (9%) (Coulam and Goodman 2000).

PROGESTERONE

Progesterone-regulated proteins include TGF β IL-1, IGFBP-1, TIMP-3, fibronectin, osteopontin, calcitonin, amphiregulin and glycodelin (Rutanen et al., 1986; Giudice 1999; Vaisse et al., 1990; Apparao et al., 2001, Seppälä et al., 2001). These cytokines, proteins and adhesion molecules have a great variety of functions at the cellular level. Progesterone may act directly or indirectly on endometrial epithelium and stroma. For example, induction of paracrine factors such as HB-EGF by progesterone may indirectly regulate other endometrial proteins such as LIF, HOXA10, $\alpha\beta3$ integrin, and DAF (Lessey et al., 2002a). Progesterone is held largely responsible for the Th-1 to Th-2 conversion in decidua, thereby contributing to the regulation of the immune homeostasis during pregnancy (Piccinni et al., 1998; 2000).

Progesterone receptor has been demonstrated in decidual NK cells (Van den Heuvel et al., 1996). The PR-positive NK cells produce an immuno-modulatory protein PIBF (progesterone-induced blocking factor) that inhibits degranulation and perforin liberation from peripheral NK cells, thus inhibiting NK cell activity (Szekeres-Bartho et al., 1985; Faust et al., 1999). PIBF has been detected in late luteal phase endometrium (Check et al., 1996), and lower levels have been reported for those women who aborted versus those who successfully completed the pregnancy (Szekeres-Bartho et al., 1989).

GLYCODELIN

Glycodelin is one of the progesterone-associated proteins, also known as PP14 or PEP (Dell et al., 1995; Seppälä et al., 1998). Glycodelin is a 28 kDa glycoprotein that belongs to the lipocalin superfamily. Stimulated by progesterone and relaxin, glycodelin becomes expressed in endometrial glands and luminal surface 4-5 days after ovulation (Taylor et al., 1998; Tseng et al., 1999; Gao et al., 2001; Seppälä et al., 2001) (Table 21.1). Glycodelin secretion into luminal fluid increases rapidly from the mid luteal phase onwards, with a short doubling time of 6.6 to 14.6 h (Li et al., 1993). Lower levels of expression in endometrium have been found in women with unexplained infertility and retarded endometrium (Klenzeris et al., 1994).

One of the biological actions of endometrial glycodelin is its immunosuppressive property, notably its inhibitory effect on the NK cell activity (Okamoto et al., 1991). During the first trimester of pregnancy, trophoblast cells invade deeply into an intimate contact with the maternal decidua. At the same time the decidua contains large amounts of CD3-, CD56+ NK cells with reduced cytotoxicity (Sato et al., 1990). It has been hypothesized that glycodelin provides temporally and spatially restricted immunosuppressive effects that protect the human embryo against these maternally derived immune cells during implantation (Okamoto et al., 1991; Clark et al., 1996). Glandular secretion of glycodelin into uterine luminal fluid results in high local concentrations, >100-fold higher than in peripheral blood. Therefore, in the reflection of endometrial secretory function the measurement of glycodelin in uterine flushings is likely to be of greater value than the measurement in serum samples (Li et al., 1993).

Glycodelin-A, isolated from amniotic fluid, is probably derived from decidualized endometrium and consists of unique complex-type oligosaccharide chains related to its biological activity, i.e. inhibition of sperm-egg binding (Dell et al., 1995). Glycodelin-S is one of the major proteins of the seminal plasma (Koistinen et al., 1996). It is immunologically indistinguishable from glycodelin-A, has the same primary structure, but is differently glycosylated and does not inhibit sperm-egg binding (Morris et al., 1996). The biological role of glycodelin-S is not known. In this context it is of interest to note that vaginal capsules containing seminal plasma have been reported to increase the implantation rate (Coulam and Stern 1995), and sexual activity during the peri-implantation period may also increase the pregnancy rate (Tremellen et al., 2000). Obviously there are many bioactive substances in the seminal plasma that could account for these observations, and it is not known whether the high seminal plasma glycodelin concentration has any role in this context. Nevertheless, these observations suggest that, in addition to fertilization, non-conceptual sexual activity may improve implantation.

LEUKEMIA INHIBITORY FACTOR

This is a 45-46 kDa pleiotropic cytokine of the IL-6 family that regulates cell proliferation and differentiation (Sentruk and Arici 1998). The activity of LIF is transmitted through LIF receptor (LIFR β) and the associated signal-transducing component, gp130. LIF expression is restricted to the luminal and glandular endometrium and is relatively low in the proliferative phase, rises after ovulation and remains high until the end of the menstrual cycle (Chen et al., 1995). LIFR β is expressed during the proliferative and secretory phases and is also restricted to the luminal epithelium (Cullinan et al., 1996). Various cytokines and growth factors induce endometrial LIF expression *in vitro*. Interestingly, *in vivo*

administration of progesterone inhibits the secretion of endometrial LIF (Hambartsoumian et al., 1998), whereas *in vitro* studies show that LIF is upregulated by progesterone and IL-4 and downregulated by IL-12, IFN γ and IFN α (Piccinni et al., 1998; 2000). LIF is probably also regulated by growth factors (Giudice et al., 1996).

LIF induces urokinase-type plasminogen activator and gelatinase, enzymes that play a crucial role in trophoblast invasion. LIF also stimulates trophoblastic hCG secretion, blocked by genistein, a tyrosine kinase inhibitor. This indicates an involvement of tyrosine kinase in gp 130-mediated hCG production. Since hCG stimulates trophoblast growth, differentiation and placental metabolism, LIF produced at the fetomaternal interface may contribute to trophoblast differentiation, maintenance of placental function and embryonic growth (Sawai et al., 1995; Sentruk and Arici 1998). Maximum endometrial LIF expression is observed at implantation, and the blastocyst contains mRNA for the receptor at this time (Charnock-Jones et al., 1994). These results and the fact that women with unexplained infertility have reduced LIF concentrations in uterine flushing suggest the importance of this cytokine in embryo implantation (Laird et al., 1997). However, a recent study on implantation points to an opposite: the LIF concentration was significantly lower in the pregnant group compared to those who did not conceive, and a low concentration of LIF in the uterine flushing at day 26 was predictive of implantation (Lédée-Bataille et al., 2002).

Functional LIF receptors (LIFR- β /gp130 heterodimers) have been found in the oocyte through early preimplantation stage embryos (Van Eijk et al., 1996; Sharkey et al., 1999), and the blastocyst formation rate is increased in embryos cultured *in vitro* in the presence of LIF (Dunglison et al., 1996). This is compatible with a trend toward decreased expression of both LIF and LIF-R β mRNAs in embryos in which growth had been arrested for 24-48 hours (Chen et al., 1999). Interestingly, when LIF can be found in conditioned medium of the embryos grown in endometrial coculture, a tendency of increased pregnancy rates have been observed (Spandorfer et al., 2001). But, transferring embryos in culture medium containing LIF does not increase the pregnancy rate in cases where LIF is not expressed in the endometrium (Borini et al., 1997).

MUC-1

Mucins are the major constituent of mucus. The cell surface mucin, MUC-1, is a high molecular mass, highly glycosylated epithelial apical glycoprotein that has both adhesive and anti-adhesive properties (Aplin et al., 1994). Its expression in glandular epithelium is transcriptionally regulated. The attaching embryo encounters epithelial glycocalyx of the endometrium that contains MUC-1. The locus of the MUC1 gene is on

chromosome 1q21 (Meseguer et al., 1998). Polymorphism has been reported for the MUC1 gene. Infertile women have a lower median allele size (2.5 kb) compared with 3.4 kb in fertile women, suggesting genetic susceptibility to infertility related to the lower allele size, possibly due to suspected failure of embryo implantation (Horne et al., 2001).

Endometrial MUC-1 is upregulated by progesterone and downregulated *in vitro* by the human blastocyst (Meseguer et al., 2001). MUC-1 contains keratin sulfate chains that disappear at implantation. At the same time the sialokeratan sulfate chains in MUC-1 increase dramatically and reach a maximum in uterine luminal fluid on days LH +6-7, the receptive period (Aplin et al., 1994; Aplin and Graham 1998). Specific glycosylation and the negative charge associated with hormonally regulated sialo- and sulfoglycans in MUC1 increase in secretory phase endometrium (Aplin and Graham 1998; Aplin 1999). Besides in the endometrial epithelium, MUC-1 is expressed in the trophectoderm of the human blastocyst. Interestingly, the presence of a human blastocyst increases endometrial MUC-1 protein at the apposition phase, whereas at the adhesion phase the blastocyst induces paracrine cleavage in MUC-1 at the implantation site (Meseguer et al., 2001). These *in vitro* experiments demonstrate that, by inhibiting cell attachment, a high density of anti-adhesive MUC-1 at the endometrial cell surface serves as a barrier to embryo attachment that must be locally removed through structural changes in MUC-1 at the implantation site, while normal expression of MUC-1 persists in neighboring cells.

L-SELECTIN-MEDIATED ADHESION

Cytotrophoblasts of ectodermal origin undergo a novel differentiation process, taking on characteristics of vascular cells (Damsky and Fisher 1998). Maternal endometrial epithelial cells upregulate selectin oligosaccharide-based ligands during the window of receptivity and, on the fetal side, trophoblast cells of an implantation-competent embryo begin to express L-selectin (Genbacev et al., 2003). In vascular biology, L-selectin enables circulating leukocytes to bind to blood vessel endothelium (Alon and Feigelson 2002). Obviously the same ligand-receptor system is functional at the fetomaternal interface, because beads coated with the L-selectin ligand 6-sulfo sLe^x bind to trophoblasts, and trophoblasts bind to ligand-expressing uterine luminal epithelium during the receptive, but not the nonreceptive, period (Genbacev et al., 2003). Thus, the trophoblasts appear to share adhesion mechanisms with leukocytes, and the apposition of the embryo during implantation shares features in common with leukocytes that roll along the endothelium before adhering and moving through the endothelial layer into the tissues (Fazleabas and Kim, 2003).

TABLE 21.1. SELECTED ENDOMETRIAL FACTORS RELATED TO IMPLANTATION

Marker	Characteristic	Reference
Steroid receptors		
ER	Maximum at mid- and late proliferative phase, downregulated in secretory phase glands	
PR	Maximum at mid- and late proliferative phase, downregulated in secretory phase glands, maintained in stroma	Lessey et al., 1988
AR	Stimulated by estrogens and androgens downregulated by progesterone,	Lessey et al., 1988 Mertens et al., 2001 Apparao et al., 2002
The IL-1 system		
IL-1 α	Epithelium	Simón et al., 1997b
IL-1 β	Epithelium	Simón et al., 1997a; 1993
IL-1R tI	Epithelium,	Simón et al., 1998a
IL-1ra	Monocytes	Polan et al., 1995
Integrins		
$\alpha 1\beta 1$	Laminin receptor, cyclically regulated from ovulation onwards	
$\alpha 4\beta 1$	Fibronectin receptor, cyclically regulated from ovulation to day 24	Lessey et al., 1994a
$\alpha v\beta 3$	Vitronectin receptor, cyclically expressed on apical pole of surface endometrium on days 20-24, requires RGD in ligand	Lessey et al., 1994a
$\alpha 5\beta 1$	Fibronectin receptor, expressed in trophoblast and endometrial stroma (constitutive), binds to RGD, e.g. in stromal fibronectin and IGFBP-1	Lessey et al., 1992 Lessey, 1997 Jones et al., 1993
The IGF system		
IGF-I	Highest expression in	

	late proliferative and early secretory endometrium, growth factor effects, stimulates $\alpha\beta\beta$ ligand binding inhibits IGFBP-1 secretion	Maile et al., 2002
IGF-II	Upregulated in late secretory phase, growth factor effects, inhibits TIMP-3 and IGFBP-1 in endometrial stromal cells	Mason et al., 1993
Types I and II IGFR	Upregulated in late secretory phase	Irwin et al., 2001
IGFBP-1	Stromal synthesis in secretory phase, downregulated by insulin,	Rutanen et al., 1986 Suikkari et al., 1988 Powell et al., 1991
	stimulated by glucocorticoids and relaxin. Increases TIMP-1, binds to trophoblastic $\alpha\beta\beta$ integrin	Gao et al., 1994 Bishof et al., 1998
		Irwin and Giudice, 1998
Glycodelin		
	Appears in glands at the onset of implantation window, secreted by pregnancy decidua, induced by progesterone and relaxin, immunosuppression at fetomaternal interface	Julkunen et al., 1986
LIF	Expressed in luminal and glandular epithelium Upregulated by progesterone and IL-4 in vitro but not in vivo Stimulates trophoblastic hCG secretion	Tseng et al., 1999 Okamoto et al., 1991 Clark et al., 1996
		Cullinan et al., 1996 Sentruk and Arici, 1998
		Piccinni et al., 1998, 2000 Hambartsoumian et al. 1998
MUC-1	Expression in glandular epithelium, with adhesive and antiadhesive properties Upregulated by progesterone. Sialokeratan sulfate	Aplin et al., 1994

chains in MUC-1 reach
a maximum in uterine
luminal fluid on days
LH +6-7

Aplin et al., 1998

L-selectin oligo-saccharide-based ligands

Upregulated in uterine
epithelial cells during the
window of receptivity.
Human trophoblasts
Express L-selectin

Genbacev et al., 2003

SPECIFIC QUESTIONS THAT HAVE ARISEN IN CLINICAL SITUATIONS

HOW CAN WE BE SURE THAT ENDOMETRIUM IS RECEPTIVE?

The embryo can implant on sites other than the endometrium, but proper placentation will not normally take place. Endometrial receptivity can be definitively established only after successful implantation, demonstrated as a positive pregnancy test and a viable fetus. Recent evidence suggests that synchrony is important, and factors that delay implantation may result in fetal loss (Wilcox et al., 1999). In non-conception cycles, there are signs and biomarkers that are expressed during the phase of the cycle when implantation normally takes place. For instance, after superovulation, endometrial thickness >7.5 mm at the time of oocyte retrieval and before ET is regarded as adequate for implantation, whereas this finding does not guarantee that implantation will take place. An increasing number of proteins have been described that may also be helpful indicators of a healthy, receptive endometrium (Lessey, 2001). Signs of non-receptivity may be more informative.

WHAT CONSTITUTES AN ADVERSE ENDOMETRIUM?

In the evaluation high-resolution ultrasonography is currently the imaging modality of choice. Before an IVF cycle ultrasound may reveal hydrosalpinges, uneven echogenicity related to polyps, fibroids or hyperplasia, and these should warrant hysteroscopy or other treatment. Endometrial cavity fluid is an unfavorable sign associated with poor ovarian response and its consequences on endometrium (Levi et al., 2001b). In a stimulated cycle, endometrial thickness of less than 5 mm on the day of hCG administration is considered unfavorable (Abdalla et al.,

1994), and the same applies to premature hyperechogenic endometrium (Fanchin et al., 2000). Here, intermediate positioning of the uterus may mislead the interpretation. The pulsatility index (PI) of greater than 3.0 as determined by transvaginal ultrasonography with color blood flow imaging immediately before embryo transfer is regarded as a sign of unreceptive endometrium (Steer et al., 1992), and the PI on the day of ET is significantly lower in those women who became pregnant as compared with those who did not conceive (Steer et al., 1995). Increased expression of ER α (Lessey et al., 2002b) and PR (Lessey et al., 1996) during the window of implantation are abnormal findings and have been reported to portend poor reproductive outcome. Circulating hormone levels may give additional information. In a stimulated cycle, high E2 levels ($>20,000$ pmol/L, or >5440 ng/L), or a high progesterone concentration (>2860 mmol/L, or >0.9 mg/L) on the day of hCG administration are regarded as detrimental for uterine receptivity (Valbuena et al., 1999).

HOW TO TREAT RETARDED ENDOMETRIUM? IS IT NECESSARY?

The classical definition of a luteal phase deficiency has rested on retarded histological maturation of the endometrium in late luteal phase (Noyes et al., 1950). Endometrial biopsies taken near the end of the cycle do not necessarily tell about maturation at the time of implantation that takes place 5-7 days earlier, and biopsies are not recommended in the IVF-ET cycle so as not to interfere with implantation. Abnormalities in the luteal phase are seen in virtually all the stimulation protocols used in IVF programs. In cycles stimulated for superovulation with GnRH agonists and human menopausal gonadotropin, a luteal phase defect is common, and routine supplementation with progesterone is recommended (Bourgain et al., 1994).

In women who will receive donor oocytes or embryos, endometrial histology is often done in a preceding cycle in order to ascertain that estrogen and progesterone supplementation is adequate. During the receptive phase, a lag in glandular development behind stromal development is common on cycle day 21, but rare on cycle day 23 (Damario et al 2001). Interestingly, clinical outcomes are virtually identical in the recipients of donor oocytes with either in-phase or dysynchronous endometrium. This is in spite of the observation that the immunohistochemical scores of markers of receptivity such as glycodelin and $\alpha v \beta 3$ integrin are significantly greater in the in-phase endometria (Damario et al 2001). Although many studies show a positive correlation between endometrial thickness and implantation, it is remarkable that oocyte recipients with a thin endometrium (<4 mm) may have normal implantation and pregnancy rates (Remohi et al., 1997). Should one wish

to do something about retarded or thin endometrium, a more effective estrogen priming has been recommended, but this may be effective for younger women only (Gleicher et al., 2000). Ultrasound examination has demonstrated that vaginal E2 administration can increase endometrial thickness, decrease the pulsatility index, and increase endometrial blood flow, but at the same time it attenuates the decrease of progesterone-induced uterine relaxation (Fanchin et al., 2001a). Therefore, adequate progesterone supplementation is important.

WHY IS IT THAT IMPLANTATION CAN BE ACHIEVED IN PATIENTS WITH PREMATURE MENOPAUSE BY SIMPLY USING EXOGENOUS ESTRADIOL AND PROGESTERONE?

The lessons learned from women with deprived ovarian function show that replacement with E2 and progesterone alone is enough for the induction of endometrial receptivity. The reason for this may be that women with no ovarian function also lack other ovarian factors, e.g. androgens that would be detrimental for implantation (see below). There is remarkable flexibility regarding the dose and the duration of estrogen/progestogen supplementation. Estrogen induces endometrial proliferation that is translated into endometrial thickness on ultrasound. Estrogen also induces progesterone receptors and, as assessed by morphometric criteria, it is the degree of estrogen priming rather than the dose of progestogen that determines endometrial responsiveness to progestogens (Gibbons et al., 1986). However, de Ziegler and colleagues (1995) have suggested that estrogen may be entirely unnecessary for endometrial maturation during the luteal phase.

The situation is different in controlled ovarian hyperstimulation (COH) of fertile-aged women in whom exogenous gonadotropins increase both estrogen and androgen secretion, so that plasma testosterone and androstenedione levels are approximately doubled by the end of COH. Estrogens and androgens upregulate androgen receptors (AR) and inhibit avb3 expression (Apparao et al., 2002). Androgens also have other deleterious effects on endometrial function, such as decreased glycodelin secretion (Tuckerman et al. 2000). Women with PCOS exhibit elevated androgen levels in serum and, consequently, endometrial AR may be increased. Excessively high estrogen concentration appears to be detrimental to successful implantation (Valbuena et al., 2001; Lessey et al., 2002b). Therefore, prevention of excessive responses in COH is the key for prevention of the concomitantly high estrogen and androgen levels. Pretreatment with oral contraceptives has been employed to suppress ovarian androgen secretion before COH, and low dose dexamethasone treatment has been tried to suppress adrenal androgen

secretion. While dexamethasone decreases baseline and post COH (day of hCG) levels of androstenedione and testosterone, the absolute increment occurring during COH remains the same, indicating that higher androgen levels result from an effect of exogenous gonadotropins on the ovary (Fanchin et al 1997). Besides androgens, there may also be other substances with adverse effects secreted by the ovary in high responders, reducing endometrial receptivity.

IS THERE ANY BASIS FOR THE THEORY THAT THE ESTRADIOL/PROGESTERONE RATIO INFLUENCES IMPLANTATION ?

This was considered important in the early days of IVF. High E2 levels increase the ratio. More recently, many IVF centers rely on ultrasound as a sole means of monitoring follicle and endometrial maturation. Special attention is then focused on poor responders and high responders. Here, serum E2 levels may give some guidance.

Clinical studies have shown decreased implantation and pregnancy rates per cycle in high responders compared to normal responders (Simon et al., 1995; Pellicer et al., 1996). A significant decrease in pregnancy and implantation rates is common when serum E2 concentrations are over 20,000 pmol/l (5440 ng/L) on the day of hCG administration regardless of the number of oocytes retrieved and the serum progesterone concentration (Basir et al., 2001). High responders also have increased androgen secretion, particularly in PCOS patients, and high androgen levels are deleterious for the endometrium. High responders often demonstrate glandular/stromal dyssynchrony with delayed glandular development and edematous stroma. It is possible that reduced glandular development and lack of glandular secretion are related to the high E2 concentrations (Basir et al., 2001). Importantly, in high responders the detrimental effect of high serum E2 concentrations on uterine receptivity has been reported from the day of hCG administration up to 7 days later (Simon et al., 1995; Pellicer et al., 1996). However, the endocrine microenvironment in the endometrium in high responders does not appear to affect embryo quality, suggested by normal pregnancy rates obtained with donated oocytes from the high responders (Simon et al., 1995). Probably the local estrogen concentration under these circumstances does not reach similar high levels as those used in in vitro studies, showing that very high estrogen concentrations decrease blastocyst formation and embryonic adhesion (Valbuena et al., 2001). Decreasing the effects of high E2 levels by an FSH step-down regimen has improved the implantation and pregnancy rates (Simon et al., 1998b; 1999). Other ways to manage the high responders include freezing of the the embryos and transferring them in subsequent cycles (Tiitinen et al., 1995). While some feel that embryo

quality is diminished by high E2 levels (Valbuena et al., 2001), new evidence based on endometrial biomarkers suggests that E2 is directly inhibitory towards the establishment of uterine receptivity (Lessey et al., 2002b) (see Endometrial integrins)

Unlike in the high responders, studies show that where a similar number of embryos with a similar number of blastomeres are transferred into IVF-ET patients and ovum recipients, COH in normal responders does not adversely affect endometrial receptivity (Levi et al., 2001a).

Premature luteinization is defined as late follicular progesterone/E2 >1 in long GnRH analogue cycles. This situation has an adverse effect on clinical outcome (Younis et al., 2001) and may be related to low ovarian reserve. A recent study indicates that progesterone arises in the adrenal cortex during the follicular phase, whereby the function of the adrenal cortex is modulated by an unknown ovarian factor, suppressed by ethinyl estradiol. There is a shift in progesterone synthesis towards the ovaries prior to ovulation (De Geyter et al., 2002).

WHAT IS THE BEST/MOST APPROPRIATE TIME TO GIVE EXOGENOUS PROGESTERONE IN DOWN- REGULATED PATIENTS?

Progesterone supplementation has been initiated on the day of oocyte retrieval (Fanchin et al., 2001b), on the day after oocyte retrieval (Propst et al., 2001), or on the day of embryo transfer (Nyboe et al., 2002). A randomized study that compares implantation and pregnancy rates in IVF patients whose intravaginal progesterone supplementation began on day 3 after oocyte retrieval or on day 6 after the retrieval showed that, in the GnRH agonist group, implantation rate is significantly decreased when the initiation of progesterone supplementation is postponed until day 6 after oocyte retrieval in the IVF-ET cycles (Williams et al., 2001). Significantly, vaginal progesterone administration starting on the day of oocyte retrieval induces a decrease in uterine contraction frequency on the day of ET (Fanchin et al., 2001b). Prolongation of progesterone supplementation into early pregnancy has no influence on pregnancy and miscarriage rates (Nyboe et al., 2002) and, therefore, the supplementation may be discontinued at the time of a positive hCG test. In keeping with this view, in women with premature ovarian failure or evidence of diminished ovarian reserve receiving donor oocytes, luteal phase supplementation has been given between days 15 to 27 (Gibbons et al., 1998).

WHAT IS THE BEST ROUTE FOR ADMINISTRATION – IS THERE ANY EVIDENCE FOR THE ADVANTAGES OF LOCAL APPLICATION AS OPPOSED TO SYSTEMIC?

Progesterone: intravaginal administration is the route of choice, and oral route is inferior. Supplementation with progesterone is preferred to luteal support with hCG because treatment with progesterone carries a smaller risk of ovarian hyperstimulation syndrome (Soliman et al., 1994). Intramuscular administration may result in higher serum progesterone levels, but intravaginal administration results in higher endometrial progesterone concentration, higher frequency of in-phase maturation, and a similar or higher pregnancy rate (Bourgain et al., 1990; 1994; Soliman et al., 1994). Intravaginal administration is also more acceptable to the women (Smitz et al., 1992; Gibbons et al., 1998; Penzias 2002), and vaginal progesterone induces profound decrease in uterine contraction frequency in estrogen-primed infertile women with premature ovarian failure (Fanchin et al., 2001b).

Estrogen: In recipients of oocyte donation, higher serum E2 levels have been observed after oral micronized E2 compared to transdermal administration, and more estrogen is converted to estrone (Krasnow et al., 1996). Vaginal administration of micronized E2 results in preferential absorption of E2 into the endometrium, consistent with the “uterine first pass” effect (Tourgeman et al., 2001a). Interestingly, intravaginal administration more effectively increases both the serum and the endometrial levels of E2 compared to the oral route (Tourgeman et al., 1999). The greater efficiency of E2 delivery to endometrium after vaginal administration makes this route an option for patients who fail to achieve adequate endometrial thickness and maturation with oral or transdermal E2 administration (Tourgeman et al., 2001b). In GnRHa/hMG cycles, no benefit has been found from routinely supplementing the luteal phase with oral E2 valerate to supplement intravaginal progesterone (Smitz et al., 1993).

IS THERE ANY IMPLANTATION WINDOW FOR HUMAN EMBRYOS?

Hatching is a prerequisite, marking the onset of implantation. The embryo modulates the endometrial microenvironment during the apposition (chemokines) and the adhesion phases (adhesion and anti-adhesion molecules) by coordinating endometrial epithelial apoptosis during the early phases of implantation (Simon et al., 1999). In clinical studies, the latest reappearance of hCG is before day LH +13, most implantations (86%) occurring between day LH +8 and day LH +11 (Liu

et al., 1995b; Wilcox et al., 1999). Importantly, in viable pregnancies implantation occurs before the corpus luteum regresses. It is likely that natural cycles are fundamentally different from cycles derived from COH or IVF. Aggressive support of the corpus luteum may obviate the need for implantation during the strict window of implantation and provide latitude for delayed implantation. Nevertheless, the important question arises concerning implantation failure during IVF cycles. It would seem that the implantation rates should be higher than they currently are.

TABLE 21.2. ELECTIVE ONE AND TWO EMBRYO TRANSFERS IN RANDOMIZED FRESH CYCLES*

	1 ET(n = 74)	2 ET (n = 70)
Pregnancies	24 (32%)	33 (47%)
Live births	22 (92%)	28 (85%)
Twins	1 (5%)	11 (39%)
Birth <37 weeks	1 (5%)	6 (21%)
Birth <2500 g	2 (9%)	10 (26%)

*From Martikainen et al., 2001.

WHEN IS THE BEST TIME FOR TRANSFER?

The extremes are pronuclear transfer and blastocyst transfer. Transfer of multiple embryos was adopted in order to increase the chances of pregnancy in a given transfer cycle. This led to a considerable increase in multiple pregnancies and prematurity-related morbidity in the offspring (Templeton and Morris, 1998). Controlled studies on elective one versus two embryo transfers have shown that, where many embryos have been obtained, elective transfer of one good quality embryo (<20% fragmentation) results in an acceptable pregnancy rate and significantly reduces prematurity (Table 21.2).

Here, transfer of multiple embryos did not increase the implantation rate per embryo. In women <36 years of age, good quality embryos that cleave rapidly (4-5 cell stage on day 2 after retrieval), high implantation rates have been observed for day 2 transfers of one embryo only. As predicted from multiple embryo transfers (Testart 1986; Lewin et al., 1994; Shoukir et al., 1997), rapid cleavers have a high implantation rate,

shown by day 3 transfers of selected single embryos that have reached a 6-8 cell stage (rapid cleavers), whereas slower cleaving embryos implant poorly (Table 21.3).

TABLE 21.3. HIGHEST IMPLANTATION RATES AFTER ELECTIVE ONE EMBRYO TRANSFER*

Day of transfer	Embryo	Implantation rate/embryo
Day 2	2-3 cell stage	9.7 %
Day 2	4-5 cell stage	35.8 %
Day 3	4-5 cell stage	0.0 %
Day 3	6-8 cell stage	45.5 %

*Adapted from Vilska et al., 1999.

The practice of elective transfer of one embryo only would require good embryo freezing facilities and allow more ET cycles in non-stimulated cycles (see Gerris, this volume). Doubts have been cast regarding this practice because of lower pregnancy rates per cycle compared to multiple embryo transfers. It is predicted that one embryo transfers will gain popularity when euploid transfers are feasible. Growing the embryos to blastocyst stage should allow more time for preimplantation diagnosis. Sequential culture media have been developed to meet nutrient requirements of the embryo as it grows and differentiates. It has been shown that implantation rates of blastocysts transferred on day 5 are higher than those of the cleavage stage embryos transferred on day 2 or 3 within the same program (Gardner 2000).

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INDEX

- Acid base 513
Acrosome reaction 442
Adhesion molecules 7
Age
 +follicular hypoxia 72
Aldosterone 89
Ammonia 309,310,
Androgens 98
Angiogenesis 17,20,21,59,60,69
 71, 72,
 +age 72
 +aneuploidy 72
 +corpus luteum 70
 +leptin 70
 thecal cells 72
 perifollicular 59
Angiogenic factors 12
 Shield 22
Angiogenin 12
Angiopoietins 1,2,12,
Apoptosis 14,211, 322,340,393
 Induced 401,402,404
 in embryos 402
 in oocytes 403
 +mitochondria 401
 +staurosporine 401,402
 +TUNEL 401
 +ROS 411
Aromatase 98
ART
 Birth defects 559
 Congenital abnormalities 557
 Low birth weight 557
 Sex chromosome defects 557
Assisted hatching (AH) 441,442,443
 444,450,457, 458,460
 Acid drilling 447
 Acid Tyrode's 444,447,454
 +cryopreservation 497
 Endogenous hCG 458
 Enzymatic digestion 445
 Fragment removal 455
 Implantation 450,459
 Indications 450
 Laser 445
 Manipulation 456
 Mechanical 444,447
 Monozygotic twinning 456
 Outcomes 446
 Partial zona dissection 443,444
 Piezoelectric manipulation 445
Poor prognosis 450,451
 Prospective study 449
 Raised FSH 452
 Thawed embryos 453
 Trophectoderm pinching 456
 Twin pregnancies 455
 Maternal age 450,451
 Zona drilling 459
Assisted Hatching
 Hardening 453
 Pronase 445,454
 Thickness 452,453
 Thinning 457,459
ATP 51,212,525
Autocrine factors 27
Autoimmune disease 465
Azoospermia 111,121,122,142
 Non obstructive 430
Basement lamina 71
BFGF 12
Biosystem 69
Binding globulins 89
 sex hormone binding globulin 89
Bioactive proteins 486
Blastocyst 172,301,303,304,306,312,
 441,485, 486,491,492,494, 495
 Bovine 491
 +Cleavage axes 559
 Hatched 577
 Hatching 443
 Implantation 443
 +zona removal 454,455
Blastomere
 Cleavage 68
 + oxidative stress 398
 lysis 391
Buffer solutions
 Bicarbonate 313,488,491
 Phosphate 488
Calcium 250
 Release 347
 Calcium signalling 258
 Wave+meiosis 345
 +sperm penetration 347,348
 +spindle 340

- Cell**
- Membrane 317
 - Tonicity 317
 - Volume 317
 - Cell cycle 209
 - Checkpoints 341
 - G1 351
 - Proteins 213
 - Regulation 249
 - S phase 351
 - Cell death 394
 - + ATP 411
 - Apoptotic cell death+
 - increased ATP 411
 - +energy supply 412
 - Necrotic cell death
 - +decreased ATP 411
 - + intracellular calcium 416
 - Oncosis 415
 - Programmed cell death (PCD) 378,379
390,393,396,401, 403,405,411
 - In vivo 379
 - Cellular homeostasis 312,319
 - CD44 19
 - Chemotherapy 164,465
 - Chemically defined media 4
 - Cholesterol+IVM 262
 - Chromatin 157,161,360
 - Condensation 560
 - +granulosa cells 213
 - Chromosome
 - Alignment 208
 - Aneuploidy 1,62,65,66,71,
171,204,343
 - +hyperstimulation 220
 - autosomal 137
 - Anomaly 242,435,487,490
 - Autosomal translocation 112
 - Balanced 112
 - +male infertility 112
 - Bivalents,separation failure 231
 - Chiasmata 205,206,209
 - Co-culture 491,495
 - Cohesion loss 209,219,
 - Congenital anomaly 487
 - Congression 211
 - +oxygen tension 212
 - Dimorphism 205
 - +FISH 232
 - Imbalance 202
 - Inversions 112
 - Maternal origin 203
 - Metaphase 231
 - Metaphase two (MII) 61,62 - Chromosome
 - Monosomy 235
 - Mosaicism 435,436
 - + spontaneous abortion 560
 - + laboratory conditions 560
 - +DAZLA 116
 - Non disjunction 203,204,205,
231,235,342
 - Number 3 116
 - Number 13 232,234,2377
 - Number 16 234,237
 - Number 18 232,234,237
 - Number 21 232,234,237
 - Number 22 234,237
 - Precocious segregation 209, 210,211
 - Polymorphic marker 203
 - Recombination 245
 - Recombination map 205,206
 - Segregation 62,63,71,203,
208,209, 219
 - Sex chromosome aneuploidy 137
 - Sister chromatid cohesion 237
 - STS mapping 141
 - Translocation 128
 - Trisomy 218,231,235
 - +age 203
 - Trisomy 15 206
 - Ttrisomy 16 203,204,206
 - Trisomy 21 204,205,206, 215,216
 - Uniparental disomy 206
 - Y chromosome 110,111,123,124,
125,131
 - Deletions, transmitted 141
 - Deletions, inherited 136,138, 141,
Yq defect 114
 - Complement regulating proteins 59
 - Conception natural 556
 - Controlled ovarian hyperstimulation
(COH) 44, 339,594
 - +Androgens 594,595
 - +cell communication 49
 - +PCOS 595
 - +pituitary suppression 49
 - Corona radiata 17, 341,442
 - retention on zona 51,56
 - corpus luteum 1,6,18,22,27,
59,70,7386,94
 - +VEGF 70,73
 - Cortical granules 347,466, 467
 - Cortical reaction
 - +ageing 561
 - Corticosteroid
 - Dexamethasone 84
 - Cortisol 87

- Cortisol
 Oxidation 96
 Cortisone ratio **94,96**
 Binding globulin **81,87,89**
- Cortisone **90**
- Cryopreservation **485**
 Ampoules 489
 Arrhenius relationship **486**
 Blastocyst **485,494,495**
 Blastomere multinucleate 492
 +Cancer 486,500
 Cold shock 469
 Cooling rates 469
 Cryoloop 495
 Cytoskeleton 486
 Embryo 466,486
 Stage appropriate 492
 Cleavage **491,492,498,**
 499,500
 Pronucleate **491,492,496,**
 498,499,500
 Extended culture 493
 Implantation rate **499**
 Thaw **491,492,494,495**
 Wastage 486
 EM grids 472
 Immature oocytes **256**
 Induced ice formation 487
 Intracellular ice 470, 472,485
 486,487
 Latent heat 487
 Metaphase II 465
 Oocyte 486,496,500
 Germinal vesicle (GV) 496
 256,465,500
 + IVM 266
 Osmotic Events **470**
 shock 470
 Ovarian tissue **466,467,469**
 Post thaw survival 471,473
 Protocols **491,494,495,500**
 Primordial follicle 473,476
 Quality control **497**
 Rapid freeze **472**
 Rehydration 490
 Seeding **487,489**
 Slow cooling **487,488,489**
 490,491,500
 Straws 472,489
 Sperm 467
 Supercooling **487**
 Take home baby rate 497
 Thawing injury 438
- Cryopreservation
 Ultra-rapid cooling **487**
 Vitrification **472,487,488,491,**
 493,495,500
- Cryoprotective agents (CPAs) **468,**
 486,487, 490
- Buffers 490
 Concentration 472
 DMSO **469,486,488,489,490**
 Ethylene glycol **469,486,488,**
 491492,494,495,496
- Glass transition temperature 471
 Glycerol **469,486,487,488**
 490,**491,492,494**
- Intracellular ice crystals **468,469**
- Intracellular proteins 468
- Membrane permeability 468
- Molecular agents 469
- Polyethylene glycol 486, 487, 488
- Propanediol **469,485,487,488,489**
 490,**491,492,496**
- Penetration 469, 471,472,473
 Temperature dependent 488
- Removal 468, 469
- Stepwise Removal 498
- Sucrose **485,490,492,491,497**
- Toxicity 500
- Hormone replacement therapy (HRT) 451
- Culture
 Blastocyst 364
 Co culture **292,292,**578****
 + assisted hatching **447**
 Condition in vitro **214**
 2 cell block 297
 2 step / sequential **292, 293**309,310,**
 311, 312**
- One step 312
 Optimal 459
 Suboptimal 367
- Culture Media
 B3 305,306
 Biological 292
 +blastocyst **335,336**
 Chemically defined **291,292,293,,**
 294,300, **312,322**
 Complex 425
 CZB **303,304,310**
 EDTA 425
 G1,G2 **310,311**
 Glucose 355,425
 Ham's F10 **291,293,294,306**

- Culture media
 Human tubal fluid (HTF) 293,
298,299,300,303, 304, 306
 +IL receptors 578
 IVF 50 310,314
 +maternal serum 399
 MEM 293,**308,309**
 Modified Mouse Tubal Fluid 309
 +oxidative stress 398,399
 P1 303,304
 Protein free 292
 Pyruvate 425
 Serum free 293
 Sequential media **425,493,494**
 Simple 425
 STF 293
 Tyrode's 295
 Whittingham's 295
- Culture conditions 368,559, 560
 Amino acids 424
 Co-culture **424,434**
 Granulosa 424
 MDBK cells 424
 Vero cells 424
- Cumulus oophorus **5,8,9,10,11, 13,14,17,259,481,525**
 +angiogenesis **74**
 +ATP generation 53
 +cytoskeletal modifications 52
 Expansion 45,51,**244,339**
 Extracellular matrix 52
 Growth factors 74
 Heterogeneity 69
 +LH Receptors 259
 Implantation marker 62,6
 Morphology^{45,51}
 +oocyte metabolism 52
 Physiological role 51
 Prostaglandins 54
 Removal
 +adhesion agonist 57
 +blastomere fragmentation 56
 +chelating agents 56
 +hypertonic shock 57
 +ICSI 45
 +steroids 54
 +transport factor to oocyte 55
 +VEGF **67,68,69,73**
- Cumulus oocyte complex
 (COC) **14,18, 47,72**
- Cytokines 59, 71,**9,11,12,86,92**
 IL1 5,21
 IL1 β 21
 TNF α 21
- GDF9 21
 TGF β 21
 Cytoplasmic halo 350
- Doppler ultrasound **59,60,61, 65,66, 68,69,74,75**
- Density gradient
 Percoll 91
- DNA
 Damage 166,319
 Heteroplasmy 207
 Polymorphism 231
- Embryo
 Aneuploidy 366,367,540,541
 Apoptosis 204,436
 Arrest 242
 Assisted hatching 433
 Laser 433
 Biochemical markers 368
 Blastocoel 366,425
 Blastocyst 234,354,426,510,
 517,527,540
 + mitochondrial DNA 283
 Blastocyst (cattle) 252
 Blastomeres + ATP 275,285,286
 Cell cycle timing 430
 Chromosome abnormalities **31,435**
 +Mosaicism 435,436
 Cleavage 233,234, **362,521,**
 530,559,
 Abnormal 362
 Axes 558
 +blastocyst 559
 +competence 353
 +Leptin 558
 +PB2 353,353
 Planes 559
 +sperm entry 349,558
 Cloning 559
 Compaction 364,425
 Competence 63,423,430
 +follicle specific conditions **417**
 +fragmentation 377, **378, 381,413**
 in vivo **379**
 + mitochondria **274,285, 283**
 Criteria 509,528,**530,543**
 Congenital abnormality **555, 556**
 +ICSI 557,559
 Cytotrophoblast 589

- Embryo**
- Day 3 362,428, 429
 - +blastocyst formation 364,428
 - Deficient culture conditions 556
 - Development arrest 423
 - Developmental competence 212
 - Eight cell 354,432
 - + epididymal sperm 430
 - Extracellular fragments 448
 - Fertilisation 558,559
 - Four cell 353,354
 - Fragments 362,428, 429,520, 521,528,530,535,542
 - Freezing 436,437
 - Frozen /thawed 522,523, 524,542
 - Gene expression 435
 - Genome activation 423,425
 - Genotype 558
 - Genetic defects 556
 - Haploidy 435
 - Hatched 426
 - Hatching 441,443
 - acid drilling 447
 - incompetence 441
 - mechanical 447
 - Heterogeneity 61,62,73
 - + ICSI 357,358
 - Implantation failure 433
 - Inner cell mass (ICM) 354,366, 426 436,456
 - L-selectin mediated adhesion 589
 - Markers 64
 - + maternal age 428,433
 - Metabolic requirements 62
 - Metabolism + mitochondria 284
 - Mitochondria 350
 - Modulators 99
 - Monosomy 435
 - Morphology 62,68,73, 367,425
 - Morphology + blastocyst 426,427
 - Mosaicism 559
 - M multinucleate blastomeres 67, 429, 520,521, 522,528,530,526, 529 , 542,543,
 - Multiple pregnancy 434
 - Multiple transfer 456
 - Normal 557
 - Nucleolar Precursor Bodies (NPB) 352
 - Oocyte quality 431
 - Ovarian reserve 431
 - Ovarian stimulation 431
 - + Oxidative stress 52
 - + paternal factors 428,430
- Embryo**
- Polyspermy 442
 - Potential 364,446
 - Preimplantation 256
 - Pronuclear 61
 - Development 52
 - + mitochondrial DNA 277
 - + mitochondrial distribution 285
 - Putative high competence, 52, 27,20 528,529,530,531,532, 533,534,535,536,537,538,539
 - Quality 60,511,514
 - Retarded cleavage 249
 - Rescue 555,556,557
 - + retarded PN formation 430
 - Selection 61,63,72,73,81, 362,505,507,513,526,532, 533,534,535,536,537,538,539
 - Sequential culture 434
 - Scoring 336,337,362, 507,512, 520
 - Stage appropriate 378,390, 432
 - Stage specific requirements 425
 - +testicular sperm 430
 - Transcription 423
 - Tetraploid 435
 - Triploidy 61,435
 - Trisomy 435
 - +vascular cells 589
 - +VEGF 69
 - Viability 63
 - Zona pellucida 426,433
 - +Pronase 433
 - Embryo Transfer 336,364
 - Single embryo transfer
 - (involuntary) 509,519,520
 - Day 2 520,524,540,541
 - Day 3 519,520,526,527, 528,540, 541,542, 543,526,528542,543
 - D3 versus D5 431,432
 - Day 5 526,528,542
 - Double embryo transfer 506507, 521,524,530,531, 532, 533,534, 536,538
 - Early cleavage 423
 - Elective single embryo transfer (eSET) 505,509, 510, 511,512, 515,517, 521,522,523,524,526, 528,530,532, 533,534, 535,536, 537,538,539
 - Multiple 334
 - Pronucleate 423,527,530,540,541, 542,543
 - 2 cell 362
 - 3 cell 363
 - Tubal transfer 448
 - Nucleoli score 426
 - Technique 510,518,519

- Embryo transfer
 Trophectoderm 54,366,426
- Embryo transfer catheter 518
- Embryogenesis 242, 337,423,558
- Endometrium 59,69,441
 Adverse 593
 Biopsy 579
 Cytokines 578
 Embryo apposition 578
 Attachment 583
 +endometriosis 580
 Epithelial apoptosis 578
 estrogen 579
 exogenous 594
 receptors 578, 593
 over-expression 580
 Extracellular matrix 578
 Fluid in cavity 593
 Function 578
 +gene inhibition 580
 Glands 587
 Glandular stroma dys-synchrony 579
 +IVF 579
 Glycodelin 586, 587
 hCG 584
 IL receptors 578
 +immunosuppression 587
 Implantation 590,591
 Luminal surface 577
 Luteal phase 580
 defects 580,581
 Morphological changes 579
 Mucins 589
 NK cells
 +implantation failure 586
 Pinopodes 580
 +progesterone 580
 Progesterone
 exogenous 594
 receptors 578,593
 Pulsatility index (PI) 593
 Receptivity 256,257,577, 592
 +menopause 594
 Retarded 593, 594
 estrogen priming 594
 progesterone supplement 593
 Secretory phase 580
 Spiral arteries 579
 Steroid receptors 580
 Stromal cell co-culture 581
 + IGF 581
 Thickness 579, 593
- Endometrium
 Th2 cells 585
- Ultrasound
 hyperechoic 579
 triple line 579
 Withdrawal bleed 257
- Endothelial cells 1,2,3,6,9,10,13,14,
 59,71,21,22,24,27,28
- Endothelin 1
 Receptors 12
- Enzymes
 3 β -hydroxysteroid dehydrogenase
 (3 β HSD) 99
 11 β -HydroxysteroidDehydrogenase
 (11 β HSD) 62,81,87,89,90,91,95
 Endogenous stimulation 96
 Endogenous inhibitor 96
 Hydrophilic stimulators 96
 Hydrophilic inhibitors 96
 Hydrophobic inhibitors 96
 Inhibitors
 glycyrrhetic acid 97
 reboxetine 97
 Modulators 99
 NADP dependent activity 96
 Freezing 91
 +IVF outcome 90,93,94,97,99
 11 β HSD1 92,94
 knockout mice 92
 Leydig cells 93
 11 β HSD2 92
 11-deoxycortisol 87
- Enzyme cofactors
 NAD 92,96
 NADPH 92,96
- Ephrins
 ET1 13,22,23,24,25,26,28
 Ephrin signalling pathways 22
- Epigenetic alteration 481
- Epithelial cells 1,3,27
- Estradiol (E2) 82,83
 basal 215
 Serum 70
 E2:P ratio 84
- Estradiol:androstenedione 84
- Estrogen
 Production 339,597
 progesterone ratio 595, 596
 uterine 1st pass effect 597
 vaginal 597
- Fallopian tube 72
- Female age 511,513,514,519,
 523

- Fertilisation 64, 162, 163,
166, 182, 343
+blood flow 60
calcium release 347
cytoplasmic halo 350
Failure 67
+meiosis 345
+ rotation of pronuclei 356
+sperm penetration 347, 348
+spindle 340
- Fetal reduction 82
- Fetomaternal interface 585
- Fertility restoration 476
- Fragmentation
- Aetiology 377, 378, 385, 386, 390
 - age related 407
 - apoptosis 378, 393, 403, 405
 - apoptotic genes 390
 - +annexin V staining 390, 392, 393,
402
 - +ATP 415
 - +blastocyst formation 382
 - +blastomere continuity 382, 383, 388
 - +caspase 403, 404
 - +chromosome anomaly 416
 - classification 377, 381
 - +cleavage 383, 385, 386
 - arrest 393
 - COMET assay 397, 398
 - +DNA damage 397, 398
 - +DNA integrity 394, 397
 - +discontinuous compaction 382
 - DNA +embryo competence 407, 408
 - elimination 390
 - Early cleavage embryos 380
 - Embryo competence 383, 385
 - Embryo selection 416
 - +energy deficiency 407
 - Fragment removal 381
 - +GV oocytes 380, 384
 - + hypoxia 379, 403, 404
 - iatrogenic 378, 379
 - +ICSI 397
 - Implantation potential 381
 - Irreversible 385, 390
 - +leptin 382
 - Lethal patterns 401, 404
 - Lysis 388
 - Membrane blebs 414
 - MII oocytes 380
 - Mechanisms 390, 391, 392, 393, 396
 - Membrane flipping 392
 - Mitochondria 392, 398, 413, 416
 - +ATP 405
- Fragmentation
- Mitochondria
- density + age 408
 - heteroplasmy 406, 407
 - inheritance 414
 - +ion concentrations 415
 - metabolism 405, 406
 - mutant number + cleavage
409
 - replication 406
 - transplantation 416
- M multinucleate 7, 8
- +Necrosis 396, 412
- Non apoptotic 405
- Nucleate 392
- + oncosis 413
- Patterns 380, 381, 382, 406, 407, 408
- +PB 393
- Phenotype+ metabolic capacity 409
- +plasma membrane integrity
393, 394
- Pronucleate embryos 380, 410
- + microtubules 415
- Pseudofragments 388
- Quantifying 377, 378, 381
- Resorption 386, 387, 388, 414
- Reversible 385, 386
- Scanning electron microscope
(SEM) 390, 391
- Spatial characteristics 380, 381, 382
385, 388, 389
- Temporal characteristics 380, 386
- Transmission electron microscope
(TEM) 391
- +TUNEL assay 394, 390, 393, 396
397
- Ultrastructure 388, 390, 395
- Fluorescence in situ hybridisation
(FISH) 204, 209, 232, 234, 235, 343
- +metaphase chromosomes 232
 - +polar body 2 234, 236
 - +Free radicals 319
- Fluorescent Alpha-satellite DNA
probes 232
- chromosome specific probes
232, 234
- Follicle 28, 29, 30, 83, 84, 87, 89
+abnormality 252
- Antral 1, 11, 44, 59, 60, 209,
252, 466, 482
- Apoptosis 216
- + in vitro culture 220
- Atresia 208, 24
- +nitric oxide 216

- Follicle**
- Autografting 478
 - Banking 468
 - Bidirectional signalling 46
 - Regulation 46
 - Blood flow 340
 - +oxygen 340
 - Cell-oocyte interactions 45
 - + cancer 264
 - +cholesterol 262
 - Cooling 466
 - Cortisol 87
 - +cryopreservation 264
 - Culture 481,480
 - +culture media 258
 - Dominant 70,**71,243,252,257**
 - +fertilisation 250,258
 - Folliculogenesis **243,244**
 - Freezing 480, 481
 - Survival 478
 - Graafian 243
 - Hormonal milieu 50
 - +ICSI 242,255,258
 - Maternal mRNA 253
 - in vivo 249
 - +meiosis **262**
 - +meiosis activating sterol (MAS) **261**
 - Multiple development 255
 - +ovarian stimulation **254**
 - +OHSS 264
 - Oxygen supply 211,212
 - pool 202,**208,214**,220,
 - Preatral 1,44,59,480,481
 - + pregnancy 253,254
 - Primary 48,466
 - Primordial 1,48,59, 212, 216,243, 338,**466**, 468,471,473,479,480
 - Reserve 465
 - Somatic cell secretion 48
 - Size + oocyte recovery **251**
 - Stimulation 44
 - +TZPS 48
 - Vascularity 243,285
 - +VEGF 212
 - +VEGF mRNA 260
 - Follicle stimulating hormone (FSH) 82,85,86
 - Step down protocol + pregnancy 596

Follicular

 - Atresia 12
 - Basement lamina 59,**72**

Follicular

 - Blood flow 60,**63,64,69,71,73**
 - +embryo cleavage 69
 - +gonadotrophins 75
 - +aspirin 75
 - +poor responders 71
 - Diameter 62
 - Dominant 11,
Heterogeneity 3,6,7,8,**11**,
61,65,66,67,74
 - +blood flow 70
 - Hypoxia 4,15,**29,30,63**
 - +age 72
 - +spindle formation 72
 - Markers of implantation 3,8, 62
 - Maturation 3,62
 - Microenvironment 70
 - Oxygenation 3,13
 - Preovulatory 86
 - Vascularity 2,3,5,6,7,8,
10,**13,14**, 60,72
 - +age **71**
 - +dissolved oxygen 61,66, 67
 - +fertilisation 65
 - grading 64
 - +oocyte aneuploidy 61
 - +pregnancy 61,63,**65,66**
 - Neovascularisation 70,**71,72**
 - Follicular fluid 1,2,3,7,11,69,91,
96,99,212,258,525
 - activin 212
 - angiogenin 212
 - +cumulus cells 259
 - +cysteine 259
 - +defined media 258
 - Insulin like growth factor 212
 - FF MAS (follicular fluid meiosis activating sterol) **214,261,265**
 - +FSH 258
 - +glutathione 259
 - + growth factors 258
 - +hormones 258
 - inhibin 212
 - inhibin B 214
 - Paracrine + autocrine processes **213**
 - Follicular vascularity
 - +oogenesis 11,13,15,46,50,70
 - gap junctions 50
 - oocyte granulosa interactions 50
 - Folliculogenesis 77
 - +oxygen supply 212
 - Frozen embryo transfer (FET) 73

- Genes**
- Co-activation 558
 - Connexin 37
 - Drosophila 132,133
 - Expression 320,321
 - gene +sterility 50
 - Gene defects
 - Single gene defects 234,234
 - Interaction 558
 - Knockout 93,94,95
 - Microdeletions 112,113,114,115,117,142,
 - De novo 115,139,141
 - transmission to offspring 111,112, 122,139
 - Molecular mapping 113,114,128,141
 - oocyte granulosa junctions 50
 - PAX6 557
 - PCR 113
 - Point mutations (small deletions) 117,118,121,138, 141
 - + fertilisation 138
 - Retroposition 127
 - RBM 125,134
 - RPS4X 134
 - Single gene defects 234,234
 - SOX3 126
 - Transcription 558
 - Transposition 127
 - ZFX 134
- Gene(s) and Male infertility**
- Absolute sequence identity 140,139
 - Amplification 124
 - Autosomal (CDYL) 128
 - AZFa 117,130,139,140
 - +USP9Y,DBY 117,121,122,130
 - AZFb +RBM gene 116,117,129,133
 - AZFc 114,117,
 - +CDY 124,128
 - +DAZ 116,124,125, 128,132,133,138,138,140,133,
 - Homologous 128
 - Homologous recombination 140
 - Homologous with Y 134
 - Introns 128
 - Male benefit genes 127, 133
 - Multiple gene action 116
 - Polygenic control 121
 - Random transposition 128
 - Recessive 141
 - RT-PCZ 141
 - RBM 124,125,129,134
- Genes(s) and Male Infertility**
- +reverse transcription 128
 - RPS4Y 134
 - Sequence tagged sites (STS) 113,114,117
 - Sex determining gene 132
 - SOX3 126,129
 - SOX9 126,127
 - SRY (male sex determining locus) 126,127,128,129
 - Testis specific 118,129
 - Transposition from autosomes 128
 - X inactivation 133,134
 - YACs 117
 - ZFY 134
 - Genetic counselling 142
 - Genome
 - Activation 244
 - Genome
 - Imprinting 253
 - Variation 243
 - Germ cell aplasia 162,163
 - Germinal vesicle (GV)
 - +mitochondrial DNA 277
 - GVBD 207
 - Glucocorticoids 87
 - GnRH+ uterine receptiivity 585
 - Gonadotrophins 62,61,69,85,242,339
 - Follicle stimulating hormone (FSH) 70
 - human chorionic gonadotrophin (hCG) 73,256,367
 - Luteinising hormone (LH) 73
 - Granulosa 1,2,7,10,11,14,17,18,27,59,66,69,70,71,86, 95,178, 249, 259,281,481
 - +COH 51
 - cytoplasmic extensions 46
 - cytoskeleton 46
 - endothelial properties 73
 - FSH receptors 259
 - Gap junctions 48,50
 - Disruption 50
 - LH receptors 269
 - Lutein cells 86,90,91,92
 - Oocyte loop 338,339,340,341
 - Oocyte interface 45,46,48
 - Oocyte signalling+IVM 54,56
 - Steroidogenic activity +age 71
 - Growth factors 46, 59,71,72,82,85
 - Epidermal growth factor 85,258,26
 - Insulin like 243
 - IGF-1 85

- Growth Factors
 PGDF 85
 Transforming growth factors
 TGF α 260
 TGF- β 85
 TGF- β 1 86
 TGF- β 2 85
 Vascular epithelial growth factor (VEGF) 7,8,9,10,
 11,12,13,67,70,86
 +follicular fluid hypoxia 72
 Receptors 16,17,18,28
- Heat shock proteins 216,322
 Henderson Hasselbach 312
 Heparin binding factors 69
 Hepatitis A,B,C 167
 HIV 167
 Hormones
 endometrial differentiation 583
 Estradiol 255
 estrogen 243
 +fertilisation 255
 FSH/rFSH 514,516,518
 FSH (follicle stimulating hormone)
 70,476
 basal 214
 +aneuploidy 214,215
 +antral follicle 215
 +Down syndrome 215
 +GVBD 262
 GnRH agonist 516,517
 glycodelin transcription 584
 Human Chorionic Gonadotrophin (hCG) 517,518,533,542
 hMG 515,516+
 LH /rLH 515,516,517
 uterine cavity 584
 Human serum albumin (HAS) 161
 HOS test 157,167,171
 Hyaluronan 18,19,20,21,22
 Hydrogen ions 301,313
 Hypertonicity 317,487
- Immune function 6, 11
 Immune system
 maternal 585, 586
 Immunoglobulins and pregnancy 586
 Implantation 577, 578
 + assisted hatching 441,450
- Implantation
 +blastocyst transfer 366
 +endometrial factors 590,591
 +estrogen 595
 + 8 cell embryos 362
 +endometrial factors 590, 591
 Failure 242,249,274
 Heterogeneity 355
 +MUC1 595
 +mitochondrial DNA 278
 +oocyte morphology 354,355
 +ovarian markers 63
 Potential 61,62, 335,368,443,
 530,540,542,
 Rate 61, 334,426,430,432,433,448
 509,510,517,520,522,524, 525,535
 536
 +seminal plasma 587
 Window 458,580,593
- Indications for treatment
 PCO 517
 Tubal 513,514
- Inheritance
 Mitochondrial 410
 +cleavage 410
 +lysis 410
 Inner cell mass (ICM) 136
 Insulin growth factor (IGF)
 +embryo 582
 System 581
 +fragmentation 581
 +stroma 581
 +stromal cell co-culture 581
- Integrins 583
 cycle dependant expression 583
 +embryo attachment 583
 expression 583
 +receptivity 583
- Interleukins 6,
 IL1 system 583
 IL 1 5,6,11,21
 IL1 β 21
 IL2 7,11
 IL6 9,11
 IL8 10,11
 TNF α 21
 GDF9 21
 TGF β 21
- Intracellular ice crystals 485,486
 487,488
- Intracytoplasmic sperm injection (ICSI)
 1,2,4,5,6,7,11,12,13,15,16,17,19
 23,34,35, 57,66 ,67,71,92,95,96,98
 +chromosome aneuploidy 135

ICSI		Male Infertility
+congenital abnormality	137,	genetic origin 111,112,113,122
557,559		+transmission to female 142
+cumulus denudation	357	Genes
+de novo balanced translocation	135,	Homologus with Y 134
136,137		RBM 125,134
+future fertility	138	RPS4X 134
Indications for	167	SOX3 126
+ IVM	242,255,258	ZFX 134
+Karyotyping	126	Immature sperm 186,195
retarded development	557	MESA 111,128
+unbalanced translocation	126	Oligospermy 111,142,136
In vitro maturation (IVM)	144,167,	Spermatocytes 116,121
241,242,247,249, 251, 252,255 ,345,		Spermatogenesis 131
255,256, 258 ,259,260,263, 340, 479 ,		Defect 117,122
480,481		Spermatogonia 116
Irreversible sterility	465	Spermatozoa
IUI	195	morphology 111,121
IVF	195,441,465	Testicular mosaicism 138
Predictors of outcome	100 ,	TESE 111
Biochemical	82	X chromosome + male
Natural cycle	94	infertility 124,125
		128,129,132,133, 141,142
Kinetochores	343,344	Malignant disease 465
Large offspring syndrome	253	Cell screening 481
Leptin	12,69	Molecular markers 479
Leukaemia	479	Maternal age 70,201,202,204,204,208,
Leukaemia inhibiting factor		281
(LIF) 587,588		+Aneuploidy 235
+oocyte	588	+chromatid errors 232
+implantation	588	+chromosome errors 235
mRNA receptors+blastocyst	588,	+chromosome constitution 219
+trophoblastic hCG secretion 588		+meiotic errors 237
Leydig cells + ageing	211	+ mitochondrial DNA 277,281
LH surge	341	mitochondrial DNA deletions 281
Ligands		+ spindle formation 218
Oocyte derived	48	Matrix metalloproteinases (MMPs) 583
Lipids		Media
inositol	250	conditioned 259
Lipid peroxidation	211	response
Liquid nitrogen		Natural 297
Storage tank	473	Optimum 297
Viral transfer	473	Concentration 296, 297,308
Live birth	162,513	Sequential simplified optimisation
		(SOM) 297,307,322
Male infertility		KSOM 297,298,300,304,307,
+aneuploidy	187,195	309,321
Gene	111,123,124,125,128	Medium supplements
		Amino acids 294,301,306,307,310
		Ca 298,299
		Cl 298
		Citrate 303
		CO2 313,314

- Medium supplements
 Dipeptide 311
 EDTA 295,301,303
 Essential amino acids 307,308,
 309,310
 Glycine 317
 Glucose 298,299,**301,**
 302,303,304,305,310,
 Glutamine 297,303,309,**318**
 K 298,299
 Lactate 294,310,314
 Na 298,299
 NaCl 297,**307,322**
 NaHCO₃ **312,313**
 Non essential amino acids 304,**308**
 309,310,
 Plasma proteins 312
 Phosphate 303
 Pyruvate 294,298,299,**301,**
 302,303
 Taurine 303,309,310
 Zwitterionic amino acids 312
 Meiosis 245,247
 anaphase 205
 arrest 245
 Follicular fluid meiosis activating sterol (FF MAS) 261,262
 Promoting complex 210
 Testicular meiosis activating sterol (T MAS) 261
 Meiosis I **206,207,209,210**
 + IVM 262
 Meiosis II 207,208,209210,
 211,217, 242, 243,245,342
 Meiotic arrest 205,211,216
 Meiotic errors 205
 metaphase II 211,212
 prophase I **205,206**
 Meiotic spindle 61,62,66,72
 Menopause 202,**215,216,219**
 +environment 215
 +ovariectomy 214
 perimenopause 202,218
 Premature 465
 +smoking 214
 +trisomies 214
 Menstrual cycle 202
 Metaphase II (MII) + nucleolar precursor bodies 352
 Methylprednisolone 447
 Microscopy
 confocal 285
 fluorescence 285
 Microscope
 Polarising light microscope
 (Polscope) 344,356,**357**
 Microtubules **343,344,349**
 organising centres 341,344
 Mineralocorticoids 87
 Mitochondria
 Anomalies in sperm 283
 Bottleneck theory 280
 DNA 211,212,274
 damage 211
 +granulosa cells 212
 Deletions in oocytes 279
 Distribution **212,285**
 Donor 275,286
 +embryo metabolism 286
 + fertilisation 273
 + germinal vesicle **277**
 + implantation failure 278
 maternal age 277
 +germ cells 276
 Heteroplasmy 275,280
 Maternal age 281
 Maternal inheritance 278
 +MII oocytes 277
 Maternal transmission 275
 Membrane potential 211
 Mutant load 274,277,
 279,280
 Mutations **278,280,281**
 + age 278,281
 clinical manifestations 278,279
 developmentally lethal 280
 in blastocysts 280
 +donor oocytes 280
 + meiosis 280
 +neuromuscular diseases 278,279
 oocyte specific 281
 +intracellular calcium 284
 +oocyte competence **275,276,277**
 organisation **284**
 Paternal DNA 275,286
 Paternal inheritance 283
 +PCR **276,277,279**
 perinuclear migration **284**
 +pH 284
 point mutations 281
 replication **281**
 sperm **282**
 +embryo competence **274,275**
 function **211**
 genes 249
 genetic defects 274
 genetic disease 274

- Mitochondria
 genome hybridization 271
 genome + oocyte
 competence 274
 Oocyte ATP content 276
 + oxidative phosphorylation (OXPHOS) 281
 paternal + embryo
 competence 284
 pronuclear embryo 275
 replication 276, 278
 transfer 207
 uniparental inheritance 273
- Mitogen 260
 VEGF 70
 Mitotic activity 494
 Mitotic spindle 498
 Molar solution 488
 Molecular stress 322
 Mono Ovulatory cycles 64
 Mucins
 +endometrium 589
 MUC-1 +implantation 589
 + blastocyst 589
- Oocyte
 Chromosome constitution 218
 Competence 45,46, 61,62
 63,73,76, 201,203, 204,206,210
 208, 212,214,218,241, 242,243,
 247,249,253, 338, 339, 340,
 342,343 , 344 ,346
 +chemical exposure 214
 + chromosome congression failure
 208
 + *c mos* 340,349
 Cooling 357
 Cortical granules 244,250
 Cortical reaction 258
 +Cumulus gap junctions 97
 +cytoplasmic ageing 214
 Cytoplasmic insufficiency 241
 Cytoplasm transfer 206,207,219,556
 + donor mitochondria 286
 Cytoskeleton 46
 Developmental potential 56,52,335
 Donation 73,201,220
 +follicular vascularity 73
 Dysfunction 556
 Endocytotic uptake 48
 Fertilisation 234, 242,244,
 345,514
- Oocyte
 genes; rec8,hrec8 209,210
 gene expression 216
 bax,bcl genes 216
 Genetic diagnosis 233
 Genetic maturation 245
 Germinal vesicle 256,352,38,346
 +aneuploidy 557
 Breakdown (GVBD) 246,247,
 248,249,251,255,262,341
 +MI 344
 +ICSI 342
 Growth 45,50,213
 +imprinting 213
 +oxygen supply 212
 +LIF receptors 588
 +co-culture 588
 Immature 241
 Imprinting disturbances 208
 Inhibition + DMAP 252
 Intracellular calcium 244,248,255
 in vitro maturation 219,258
 +LH receptors 254,339
 +MAP kinase 349
 Manipulation 55
 +metaphase arrest 211
 Maturation promoting factor (MPF)
 247,248
 Maturation 51,87,242,244249,
 250,518
 +polar body 356
 Meiotic maturation +
 Mitochondria 275
 Mitochondrial DNA deletions 279
 Microtubules 284
 Programming 249
 Mechanical manipulation 45
 Meiosis I 238
 Meiosis II 231,232,237,242
 Meiosis phase promoting factor
 (MPF) 340
 Meiotic arrest 55,339,340
 Immaturity 44
 Recombination 231
 errors 236,237,
 Metaphase II 231,237,242
 245,246,342
 +aneuploidy 231
 +aneuploidy rate 234,234
 +ATP 407
 + chromosome abnormalities
 234
 +complex errors 237
 +chromatid errors 235,237

- Oocyte
 +FISH **232**
 +pregnancy **407**
 + smoking **214**
 +spindle **343,344,345**
 Microenvironment **70**
 Micromanipulation **233,234**
 Mitochondria **338,346,362, 559**
 + mitochondrial genome **274**
 +ovarian stimulation **255**
 + PCO **257**
 +poor responders **215**
 Transfer **556**
 +ageing **351**
 +cell cycle control **352**
 +fragmentation **416**
 Morphology **63,67,**
 mRNA **249,423**
 nuclear +cytoplasmic maturation
338
 Nuclear + cytoplasmic synchrony
252
 nuclear /cytoplasmic
 transplantation **275,28**
 +ICSI **283**
 + live births **275**
 Nuclear exchange **219**
 Nuclear maturation **242,247**
 Nuclear maturity **62,66**
 Nuclear normality **236**
 Nuclear transfer **206,207**
 Oxidative damage **28**
 Parthenogenesis **246**
 Parthenogenetic activation +
 Perinuclear microtubule organising
 centres **284**
 Perivitelline space **51**
 Polymorphism **214**
 Polyspermy **234,244**
 Polyspermy block **250**
 Primordial **276,468**
 Proteins **211**
 puromycin **231**
 + respiration **212**
 Quality + maternal mRNA **251**
 Retrieved **517**
 RNA synthesis **338**
 Selection **357**
 Size **251**
 Somatic cell signalling **217**
 Specific gene expression **48**
 Sperm binding **250**
 Sperm entry site **357**
- Oocyte
 Spindle **61,63,67,245,**
246,340,341,342,343
 Abnormalities **343,344**
 +cryopreservation **343**
 oocyte age **345**
 Viability – molecular markers **251**
 Oocyte - cumulus
 paracrine exchange **53**
 Oocyte - granulosa
 adhesion junctions **46**
 adhesin – cadherins **48**
 calcium dependent **50**
 cell junctions **46**
 gap junctions **45,46,55,57**
 connexin subunits **46**
 interactions **46,47,51,55**
 +ovulation **51**
 interface **48**
 microenvironment **60**
 signalling **54**
 Oocyte - somatic cell
 contact **45**
 interactions+embryo formation **53**
 removal **51**
 Oogenesis **337,558**
 + age **203**
 Oral contraceptives **595**
 Organic osmolytes **318,319**
 Osmolarity **296,300,317,319**
 Osmotic
 forces **486**
 shock **487,489**
 Ovary **59**
 ageing **217**
 Banking **468**
 Cortex banking **468**
 Embryonic **205**
 Ovarian reserve **202**
 Resistant **72**
 Physiological age **218**
 Thecal cells **59,60,69,71**
 Ovarian
 Ageing **70**
 Angiogenic growth factors **477**
 Apoptosis **216,217**
 Autografting **466, 476, 477,478**
 Blood flow **60**
 Biopsy **44**
 Cooling **468,469**
 Cortex **466, 478,479**
 Cryopreservation **50,466, 67,470,**
471,472,481
 Slow freeze **471**

Ovarian		Polar Body	
Dehydrogenase		+ 2 cell embryo	362
stimulators	98	Polar Body 1 (PB1)	45,232, 233
Inhibitors	98	234,235,237,239,245,342,345,346	
Failure	465, 466	abnormality	342
Grafting	481	Aneuploidy free	238
+revascularisation	481	degradation	342
Metastases	481	+meiosis	341
Pedicle	477	+mitochondria	346
Reserve	215	Polar Body 2 (PB2)	234,234,236,
Response	60	345,348, 349,350	
Stimulation	505,517,518	Abnormality	236
Tissue	466,485,500,501	+cleavage plane	350,353
Tissue cryopreservation	469,500	degradation +competence	354
+Autologous transfer	500	+FISH	233,
+live births	477	+trophectoderm	354
Dissociation	469	+TUNEL	404
Thawing	469, 470	Polycystic ovarian disease (PCO)	
Xenografts	476,477	254,256,257,258	
Vascularity	60	+fertilisation	257
Vascularisation	+antioxidants	+ IVM	253,264
+vitamin E	477	+ pregnancy	255
Ovarian Hyperstimulation Syndrome		Polymerase chain reaction (PCR)	233
(OHSS)	8,9,86,518,521	Polyvinylpyrrinodole (PVP)	167
+ IVM	264	Pregnancy	586
Ovulation	477	+age	71
irregularities	202	biochemical	535,536
Oviduct / oviductal fluid	296,299	Cumulative rate	61
300,301,311		Failure, repeated	452
Epithelial cells	424	Maximising	506,508
Ovulation induction	44	Multiple	65,71,334
Oxygen tension	316	Optimising	508,510
Patient		Rate	66,167,168, 336,433,448
counselling	543	507,511,512,518,521,522,	
selection	505,523,525,	527,534,535,537,539	
	526,538	Twin	506,507,508,511,512,513,521
Placenta		526,534 ,580	
+mitochondrial DNA	283	Triplet	507,508,511,512
Perivitelline fluid	301	Multiple	505,507,508,511,512,
Preimplantation genetic diagnosis		514,521, 535,536,543	
(PGD)	219,234	+follicular vascularity	64,65
pH	301,312,313,314,315,	Premature ovarian failure (POF)	
Plasma membrane	486,487,489,490	216,217,220	
Polar body	525	+chemotherapy	217
biopsy	204,218	Preimplantation genetic diagnosis	
formation	208	(PGD)	560
Genetic analysis	209, 238	Progesterone	69,70, 586
+ICSI	356,357	Exogenous	596
Morphology and competence	356	+pregnancy	596
+ oocyte maturation	356	route of administration	597
Removal	233	vaginal	596
		Regulation of homeostasis	585
		Pituitary downregulation	62,64,72, 73
		Programmed cell death (PCD)	378,379

- PCD **390,393,396,401,**
402, 403,405,411
- In vivo **379**
- Prolactin **82,85**
- Pronuclei **348,350,520**
- Alignment + developmental competence **353,356**
 - Aneuploidy **359**
 - Arrest **350**
 - Breakdown **349**
 - Chromatin **352**
 - Female **349**
 - Formation **250,251**
 - Inequality **360**
 - Male **349,350**
 - + delayed rotation **360**
 - Parthenogenesis **349**
 - Scoring **218,335,336,358**
- Proteins
- Adhesion **578**
 - Protein kinases **207,219,340,341**
 - MOS kinase **208 211**
 - MAP kinase **208,211,342,349**
 - MISS **208**
 - Mitogen activating protein (MAP) **248**
 - ribosomal protein kinases **211**
 - Synthesis **249**
 - Tyrosine kinase **46**
 - Purines **248**
 - Adenosine **248,249**
 - Hypoxanthine **248,249**
- Quality control **556**
- Radiotherapy **164**
- Reactive oxygen species(ROS) **211, 281,**319****
- +cancer **281**
 - +infertility **281**
- RedOX **424**
- Reproductive senescence **279,281**
- Ribonucleoprotein synthesis **351**
- RNA
- Damage **320**
 - mRNA **322**
 - mRNA transcript **320**
 - polymerase **558**
 - transcription factors **558**
- Salting out **486,487**
- Semen
- Colour **152**
 - Composition **157**
 - (ROS) reactive oxygen species **157,158**
 - volume **152**
- Serum
- Autologous **83,490**
 - Human fetal cord serum **490**
 - Maternal **257**
 - Pooled serum albumin **488,490**
 - Substitute **488,490**
- Sperm
- Acrosome **167**
 - Analysis **151**
 - +aneuploidy **179**
 - Azoospermia **162,163**
 - Binding **346**
 - borne oocyte activating factor(SOAF) **348**
 - centrosome **349**
 - chromatin **171**
 - chromosome anomalies **163**
 - chromosome aberration + ICSI **196**
 - +creatin-N-phosphotransferrase **180**
 - +creatine Kinase (CK) **180**
 - 180,181,182,**198****
 - Decondensation **250,348,349**
 - Diploid **171**
 - Donor **163**
 - Ejaculate **162,163**
 - Epididymal **162**
 - + blastocyst formation **430**
 - +DNA degradation **179**
 - +Immunocytochemistry **191**
 - +IUI **182**
 - Maturity **172**
 - Morphology **155,156,171**
 - Anomalies **283**
 - strict Kruger **156**
 - + mitochondrial DNA **282**
 - +prohibin **283**
 - Motility **157,161,171**
 - Nuclear maturation **161**
 - +Plasma membrane remodelling **182, 183,195**
 - Selection **172**
 - +spermiogenesis **183**
 - Spindle **211**
 - abnormalities **209**
 - + age **356, 357**

- Spindle**
- assessment **357**
 - centriole **207**
 - dysgenesis **211**
 - + fertilisation **356**
 - formation **207**
 - integrity **356**
 - tubulin regulation **207**
 - microtubules **207**
 - motor proteins **207**
 - morphology **208,218**
 - motor proteins **207**
 - multiple acentriolar microtubule organising body **207**
 - polar microtubule organising centres **207**
 - position **356**
- Terrminology** **155**
- Testicular + blastocyst formation **430**
- Viability** **164**
- WHO values **151**
- +zona pellucida binding **181,183,196**
- +CK **182**
 - Binding sites **182**
- Sperm Cryopreservation** **163,167,467**
- Anisotropy **166**
 - Cryodamage **165**
 - Cryophysical behaviour **163**
 - Freeze thaw **164,166**
 - Glass vials **167**
 - Indications for **164**
 - Intracellular ice **165**
 - Iso osmotic volume**165**
 - Media **165**
 - Slow cooling **164,165**
 - Slow freeze **163**
 - Straws **167**
 - Testicular sperm
 - Quarantine **167**
- Sperm function**
- Biochemical markers **179,180**
 - HspA2 **179,180,181, 182,183, 184,195**
 - +cytoplasmic retention **180**
 - +pregnancy **182**
- Sperm maturity**
- +aneuploidy **179,181,184,185, 186,198**
 - +cytoplasmic retention **179,180,181, 182,183,191**
 - +FISH **188,191,196**
 - +autosomal aneuploidy **188**
 - +sex chromosome aneuploidy **188,191**
- Sperm Maturity**
- +lipid peroxidation **181**
 - Sperm preparation **161,179**
 - +aneuploidy **184,187**
 - Density gradient **157,158,159,166 179,184,186,187,192,195,198,199**
 - Glass wool filtration **157**
 - Hyaluronic acid (HA) **179**
 - sperm binding **179,181,195,196**
 - Binding for sperm selection **195**
 - Bound sperm + disomies **187,189**
 - Bound sperm and diploidy **187,189**
 - +ICSI **179**
 - +maturity **196**
 - +sperm motility **195**
 - +sperm selection **196,199**
 - +immature sperm **184,186,197, 192,195**
 - Isolate **184**
 - Percoll **184**
 - Pentoxyfylline **167,171**
 - Swim up **158,166,179,198,199**
 - +aneuploidy **188,190,191,192,192**
 - +diploidy **187,191,192,195**
 - +sperm selection **190,191**
 - Wash **158**
 - Sperm penetration
 - KIT ligand **339**
 - +spindle wave **347,348**

Sperm protein

 - HSP70-2 **184**
 - Gene + maturation arrest **184**
 - +meiosis **184**
 - +testis expression **184**
 - in spermiogenesis **184**
 - sperm selection+HA **196**
 - Spermatids **161,166,167,172**
 - Spermatogenesis
 - +aqe **203** - Spermiogenesis **180,181,183,186**
 - Developmental defect **180,207**
 - + temperature control **357**
 - Spontaneous abortion **203**
 - Stem cells **477**
 - Steroidogenesis **477**
 - Steroidogenic activity **59,72**
 - Stimulated Intrauterine Insemination (SIUI) **64,71**
 - Surrogate mothers **324**

TEK and TIE receptors **13,14 ,16,28**

- Teratogen 486
 TESE 160,162
 Testis
 Biopsy 163
 Leydig cells 93,161
 Sertoli cells 161,162,163,166
 Specific proteins 182
 Sperm 160
 Testosterone 93
 Thermal
 conduction 488
 exchange 487
 Transzonal processes (TZPs) 47,48,
 52,57
 Cytoskeletal elements 52
 Endosomes 48
 Gonadotrophin dependent 52
 + IVM 52
 + Leptin distribution in oocytes 53
 lysosomes 48
 microfilaments 47
 microtubules 47,52
 mitochondria 47,53
 organelle movements 52
 +STAT3 distribution in oocytes 53
 ultrastructure 47
 Trophectoderm 336
 Trophoblast 577,585
 Tubulin 343
 +age 343,344
 Turner syndrome 216
 Twin prone patients 508,514,520,538
- Ultrasound 61, 339,510,579
 Colour power Doppler 61, 69,74,75
 Doppler 59,60,65,66,68
 Power Doppler 60,73,74
 Resistance index (RI) 60,70
 Pulsatility index 70
- Uterine
 artery 60
 blood flow 60,579
 Contractions 432
 Fluid 298,299,311
 Markers 579
 +maternal age 201
 Receptivity 201,433 ,458
 RI 60
- Vascular leak syndrome (VLS) 8
 Wound healing 7,20
- Zona pellucida 46,486,48 ,167, 250
 301,38,353,466,442,,493,497
 Acid digestion 442,444
 Assisted hatching 560,561
 Block to polyspermy 442
 Bound factors 55
 Breakers 443
 +cortical reaction 250
 Criteria 561
 + cryopreservation alterations 453
 Culture media 443
 Damage + cryopreservation 454
 Defects-embryo specific 443
 Drilling 459,560,561
 Filaments 442
 Glycoproteins 442
 Hardening 258,561
 Lysins 443
 Manipulation 441
 Partial zona dissection (PZD) 443
 444,448
 Remodelling 48
 Removal 454,455
 Sperm binding 442
 Structure 442
 Thickness 442,448
 Thinning 442,443
- Zygote
 Cytoplasmic halo 299,350,361,
 Score 350